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ENDEMIOLOGY OF CANCER OF THE LUNG IN ENGLAND AND WALES.*

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Concept of endemiology in connection with cancer.

The general idea that irritants, poisons or deficiencies may be important factors in the pathogenesis of cancer of deeply seated as well as of superficial organs is hardly a new one. Indeed, early in the 19th century a careful local study of cancer deaths in Verona was made by Stern (1842), the notion of cancer houses was looked into and fragmentary studies of cancer endemiology were made in Switzerland, America and elsewhere. The concept of cancer as a unitary disease affecting organs rather at random has tended to discourage detailed research on site distributions.

The General Register Office in London worked patiently upon the occupational aspects at successive census periods with increasing detail as death certification of primary cancer sites improved. Recently the Danish Cancer Registry, initially rather sceptical of some of the conclusions reached, has admitted that Danish data tend to confirm them (Clemmesen, 1951), and similar data have been published from the Netherlands (Versluys, 1949). In 1950 and 1951 the Council for Co-ordination of International Congresses of Medical Sciences arranged conferences on what was at first called "geographical pathology", but has now become "endemiology" of cancer. Very little has been written, however, on lung cancer from this aspect except with respect to tobacco, although it came under discussion at the first of the above-mentioned conferences (C.C.I.C.M.S., 1951; Daff, Doll and Kennaway, 1951).

In the present paper the short term "lung cancer" is used to mean carcinoma and other malignant neoplasm of the bronchus, lung and pleura; and when "mortality" is mentioned it is understood that whatever rate or index is used has been standardized for age.

* The substance of this paper was communicated to the Metropolitan Branch of the Society of Medical Officers of Health on May 9, 1952.

Trend of death-rates attributed to lung cancer in England and Wales.

Table I gives the mean annual death-rates per million living at different ages for each sex in six periods of years from 1911–20 to 1945–49 in England and Wales as a whole. Rates for years prior to 1940 have been corrected to allow for the change made in that year in the method of selecting the underlying cause of death; and those for males in 1945–49 relate to civilians only. The rates are based, of course, on statements made on death certificates, and they

TABLE I.—*Death Rates per Million Living from Cancer of Lung and Bronchus in England and Wales*, 1911–1949. *Ages 25 and over.*

	1911–20.	1921–30.	1931–35.	1936–39.	1940–44.	1945–49.	Ratio of 1945–49 to 1936–39.
Males:							
25–	5	7	14	23	20	31	1·35
35–	13	28	68	99	130	168	1·70
45–	33	72	215	335	453	700	2·09
55–	61	124	346	579	866	1412	2·44
65–	72	133	344	569	799	1456	2·56
75+	39	87	235	381	498	844	2·22
Females:							
25–	2	3	5	6	8	11	1·83
35–	8	10	18	23	28	36	1·57
45–	19	24	46	60	72	94	1·57
55–	32	48	92	122	140	196	1·61
65–	39	58	123	177	205	307	1·73
75+	26	49	104	155	183	275	1·77
Ratio of male to female rate:							
35–	1·6	2·8	3·8	4·3	4·6	4·7	
45–	1·7	3·0	4·7	5·5	6·3	7·4	
55–	1·9	2·6	3·8	4·7	6·2	7·2	
65–	1·8	2·3	2·8	3·2	3·9	4·7	
75+	1·5	1·8	2·3	2·5	2·7	3·1	

TABLE II.—*Ratio of Male to Female Death-rates from Cancer of Lung and Bronchus at Various Ages in* (a) *County Boroughs*, (b) *Rural Districts, of England and Wales*, 1921–39 and 1940–46.

Age group.	County boroughs.		Rural districts.	
	1921–39.	1940–46.	1921–39.	1940–46.
0–	1·9	2·1	2·3 } 2·6	0·6 } 2·1
25–	2·9	2·5	3·1 }	2·5 }
35–	4·3	4·8	2·7	4·2
45–	4·7	7·3	2·9	4·6
55–	4·2	6·8	2·4	4·4
65–	3·0 }	4·2	2·3 }	2·8
75+	2·5 }		2·2 }	

indicate that during the 10 years preceding 1945–49 the rate of frequency of such statement at ages over 35 increased more rapidly amongst men than women, and that the increase was relatively greatest amongst men between ages 55 and 75. In 1911–20 certified mortality of men was about 1·7 times that of women, but the sex-ratio increased steadily at each age group over 35, until in 1945–49 it exceeded 7 at ages between 45 and 65, and reached 4·7 at 35–44 and 65–74.

Table II shows the sex-ratios in two periods in the County Boroughs (i.e.,

large towns other than London), and in the rural districts. At ages under 35 the ratio was virtually the same in each class of area and did not increase. At ages between 45 and 65 it was considerably greater in the towns than in rural areas in both periods of time, and it increased in both types of area between 1921–39 and 1940–46.

Table III shows that the apparent effect of urban environment was more pronounced amongst men than women, particularly between ages 45 and 75.

TABLE III.—*Ratio of Death-rates in County Boroughs from Cancer of Lung and Bronchus to those in Rural Districts of England and Wales*, 1921–39 and 1940–46.

Age group.	Males.		Females.	
	1921–39.	1940–46.	1921–39.	1940–46.
0–	1·2	1·9	1·5 } 1·8	0·6 } 1·8
25–	2·0	2·8	2·1 }	2·7 }
35–	2·5	2·0	1·6	1·8
45–	2·6	2·1	1·6	1·3
55–	2·4	2·3	1·5	1·5
65–	2·1 }	1·9	1·6 }	1·3
75+	1·7 }		1·5 }	

Lung cancer mortality in counties and towns during 1921 to 1930.

Fifteen years ago I called attention in the annual reports of the British Empire Cancer Campaign to the distribution by counties in England and Wales of mortality from lung cancer according to death certificates in the decade 1921–30 (Stocks, 1936, 1937, 1939). Black patches appeared on the map in London and neighbouring counties, the West Riding and Nottingham. In a monograph from the General Register Office in 1947 I pointed out that there was an inverse correlation between the annual amounts of sunshine recorded in 20 large towns and their lung cancer mortalities; and, after allowing for the effects of latitude, concluded that "the only explanations . . . which seem adequate are that either smokiness of atmosphere is an important factor in itself in producing cancer of the lung or sunshine is an important factor in preventing its incidence" (Stocks, 1947). At that time I still had to work upon the rates for 1921–30, and failed to find any significant correlation either in the large towns or in the Metropolitan Boroughs between lung cancer mortality and the social class or overcrowding indices.

Recent research on correlation with tobacco smoking.

Between 1930 and 1946 the recorded rates in England and Wales as a whole had been rising steadily, and to a surprising extent even when increasing use of X rays was considered. Consequently the Medical Research Council called a conference to advise whether any useful research could be undertaken to throw light on the causes. The outcome was the appointment of a small working party to initiate a statistical study of tobacco-smoking histories of lung cancer patients and of controls. The result of that study was published in September, 1950 (Doll and Hill, 1950), and in my view it proved beyond doubt, in conjunction with American evidence of the same kind (Wynder and Graham, 1950) that

tobacco smoking is a very important factor in the causation of lung cancer. It did not prove, however, that tobacco smoking is the only important factor in causation. It has been suggested that the figures could mean that 90 per cent of the lung cancer occurring in Greater London is caused by tobacco; but they do not necessarily mean that, and there are reasons for thinking that the proportion is much lower, and that atmospheric pollution may be an additional factor. These reasons derive from some statistical facts not hitherto published.

Mortality in London in 1946 *to* 1949.

In the four years 1946 to 1949 the deaths of males attributed to lung cancer in England and Wales averaged 8183 annually, and of females 1774. In 1950 deaths of males exceeded 10,250 and those of females were just short of 2000, and in 1951 the total reached 13,000. The numbers show no sign of levelling out, and were increasing by about 8 per cent annually during the period 1946–49.

TABLE IV.—*Cancer of Lung and Bronchus :* Mean Annual Death Rates by Sex and Age in England and Wales* 1921–30 *and* 1946–49, *and in London* 1921–30.

Age group.	England and Wales, 1946–49. Population at end of 1947 (thousands). Males.†	Females.	Deaths from cancer of lung, etc., in period. M.†	F.	Mean annual death rates per million. M.†	F.	1921–30. Mean annual death rates per million.‡ England and Wales. M.†	F.	London. M.†	F.
0–	4,710	4,518	31	18	2	1				
15–	2,269	2,930	88	35	10	3	3	1	5	1
25–	3,067	3,291	349	143	28	11				
35–	3,276	3,414	2,187	507	167	37	29	10	54	18
45–	2,627	2,991	7,618	1,157	725	97	73	25	156	35
55–	2,007	2,460	11,840	1,964	1,475	200	128	50	257	85
65–	1,368	1,776	8,417	2,251	1,538	317	136	60	222	94
75 and over	564	888	2,017	1,022	894	288	92	51	206	92
All ages	19,888	22,268	32,547	7,097						

* Includes pleura. † Civilian males. ‡ These rates have not been corrected as in Table 1.

If we go back 22 years, work out the death rates of males at each age period in the decade 1921–30 and apply them to the populations of corresponding ages at the end of 1947, the calculated annual deaths of civilian males in 1946–49 would have been 812, so male mortality as given by death certificates increased 10-fold in the 22 years. The national rates at different ages are compared in Table IV, which shows that at ages 35–45 the increase was 6-fold, and at 55–75 it was more than 11-fold.

How much of this enormous increase has been due to more complete detection of the disease and how much to increased incidence we can only surmise. For London County as a whole, allowing for age changes, male mortality increased about 8-fold between the two periods; and it is now about 60 per cent greater than in the country as a whole, and 2½ times the rate for rural districts. Detailed

TABLE V.—*Cancer of Lung and Bronchus :* Mortality in London according to Death Certificates in* 1921–30 *and* 1946–49.

Part of London.	Area in acres (hundreds).	Persons per acre.		Number of deaths registered.				Comparative mortality figures.		
				1921–30 Ages 45 and over.		1946–49. All ages.		Index(1) 1921–30 (persons).	Calculated deaths(2) 1946–49 (males).†	C.M.R.(3) 1946–49 (males).†
		1931.	1951.	M.†	F.	M.†	F.			
Battersea	21·6	74	54	34	13	153	31	89	84·7	181
Bermondsey	15·0	74	40	31	13	89	13	122	42·8	208
Bethnal Green	7·6	142	77	39	11	97	24	147	43·1	225
Camberwell	44·8	56	40	53	18	245	46	78	128·1	191
Chelsea	6·6	89	45	24	7	39	7	119	32·7	119
Deptford	15·6	68	48	29	8	99	23	97	58·4	152
Finsbury	5·9	119	60	17	6	57	13	92	27·2	209
Fulham	17·1	89	72	42	18	138	18	109	91·4	151
Greenwich	38·6	26	24	14	3	91	9	50	59·9	152
Hackney	32·9	66	52	57	32	204	48	121	125·3	163
Hammersmith	22·9	59	52	35	11	162	27	100	85·6	189
Hampstead	22·7	39	42	26	14	88	31	120	68·6	128
Holborn and City	10·9	40	28	11	4	43	12	68	26·4	163
Islington	30·9	104	76	68	36	315	56	91	171·0	184
Kensington	22·9	79	73	38	21	131	42	94	109·6	119
Lambeth	40·8	73	56	76	28	258	67	96	166·6	155
Lewisham	70·1	31	32	37	24	226	49	92	165·6	136
Paddington	13·6	107	92	34	22	144	36	110	88·8	163
Poplar	23·3	67	32	45	7	94	21	102	52·6	180
St. Marylebone	14·7	66	51	35	10	85	32	118	59·7	142
St. Pancras	26·9	74	51	48	19	196	30	88	113·2	173
Shoreditch	6·6	148	68	27	7	70	11	106	32·3	217
Southwark	11·3	152	86	47	14	133	22	100	72·9	182
Stepney	17·7	128	56	85	28	149	22	153	76·5	195
Stoke Newington	8·6	59	57	18	2	60	8	111	32·9	182
Wandsworth	91·1	39	36	67	32	350	110	79	255·3	137
Westminster	25·0	52	39	45	16	102	29	115	82·3	123
Woolwich	82·8	18	18	27	11	131	23	78	117·1	112
London	748·5	59	45	1,108	438	3,949	860	100	247·1	160

* Includes pleura.

† Excludes non-civilian males both from deaths and population.

(1) Per cent ratio of registered deaths at 45 and over to number calculated when London rates at 45–, 55–, 65–, 75+ are applied to the populations at those ages.

(2) By applying national rates at 0–, 35–, 45–, 55–, 55–, 65–, 75+ to the populations at those ages.

(3) Per cent ratio of registered to calculated deaths.

figures for the Metropolitan Boroughs are given in Table V, and Fig. 1 shows the mortality distribution in 1946–49. For 1921–30 there were two areas of specially high certified mortality, one in Bermondsey, Stepney, Bethnal Green, Hackney and Stoke Newington, the other in Hampstead, St. Marylebone, Paddington, Westminster, Chelsea and Fulham, an area which probably contained at that time the best diagnostic facilities for lung cancer. In 1946–49 mortality of males was apparently highest in Bermondsey, Stepney, Bethnal Green, Shoreditch and Finsbury, and rather high in the adjacent boroughs of Islington, Stoke Newington, Poplar, Southwark and Camberwell, and also in Battersea and Hammersmith.

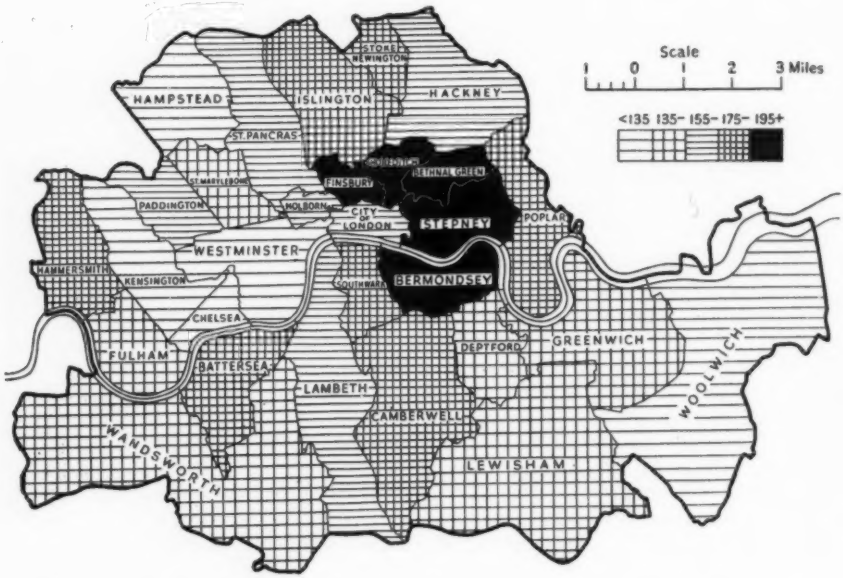

FIG. 1.—Cancer of lung, bronchus, pleura. 1946–49 deaths of males per 100 (calculated by applying rates for England and Wales to populations at ages 0–, 35–, 45–, 55–, 65–, 75+) in the City of London and metropolitan boroughs.

But in the West End area extending from Hampstead to Fulham mortality figures are now below average, and in my opinion this change is due to virtual disappearance of the diagnostic differential for lung cancer within London, so that the map now indicates mainly differences of incidence instead of a confused mixture of the incidence and diagnosis effects.

What is the explanation for rates ranging from 195 to 225 in Bermondsey, Stepney, Bethnal Green, Shoreditch and Finsbury, contrasted with about 136 in Lewisham and Wandsworth, 125 in Chelsea, Westminster, Kensington and

Hampstead, and 112 in Woolwich ? The differences are large, and it is obvious that they are positively correlated with the old social class and density-per-room indices based on the census of 1931, but that does not explain them. The occupational mortality of 1930–32 showed no social class gradient for lung cancer as recorded on death certificates ; but Clemmesen and Nielsen have reported recently (1951) that in Copenhagen such a gradient has now appeared, and there is little doubt that the census analysis in this country will eventually reveal the same thing. Whatever the reason may be, it can hardly be supposed that the people in North-East London smoke 50 per cent more tobacco than those in South-West London, though they might tend to smoke different brands.

Mortality in other large towns in 1921 *to* 1939 *and* 1946 *to* 1949.

Turning to the County Boroughs, comparative mortality ratios have been calculated for the 19 years 1921–39 for lung cancer by applying the death rates at separate age groups in all County Boroughs combined to the 1931 Census populations at the same ages for each town, and are shown in Table VI. For males the 11 towns with highest mortalities at that time, all with statistically significant excess over " expectation ", were Manchester (182), Leeds (169), Liverpool, Nottingham, Smethwick and Sheffield (144–149), Birmingham, Salford, West Ham, Bootle and Southend (130–138). Of these, 9 were in or served as dormitories to large conurbations, and the other two also had large populations. Of the next 12 in descending order, Halifax, East Ham, Croydon, Birkenhead, Huddersfield, Wallasey, Wolverhampton, Bradford and Stockport were in large conurbations, the other three being Hull, Swansea and York. At the lower end of the list were 13 towns with ratios below 50, of which only South Shields was in a conurbation. For females the order was much the same, 8 towns having ratios over 130 being, in descending order, Bootle, Leeds, Birmingham, Huddersfield, Manchester, Sheffield, Chester and Liverpool ; whilst at the lower end were 13 towns, mostly small, with ratios less than 50.

This curious relation with size of continuous urban area suggested a similar study of London and the County Boroughs for the most recent period of 1946–49, using the local populations by ages as ascertained by the Registrar-General at the end of 1947 and applying England and Wales rates at 6 age groups to them to calculate " expected " deaths. In Table VI, wherever two or more towns or London were actually contiguous like Manchester, Stockport and Salford they have been grouped into a single area, and the 70 resulting units arranged in descending order of the number of occupied dwellings at the last census, the present population densities per acre being also given. The reason for this arrangement is that amount of atmospheric pollution by smoke from dwellings must be some function of (1) the total area over which dwellings are spread more or less continuously, (2) the density of dwellings per acre, and (3) the meteorological behaviour of the air as a whole.

Correlation with extent and density of built-up area.

If lung cancer is caused in part by atmospheric pollution through smoke from domestic chimneys the rate would be expected to increase as the total number of inhabited dwellings in the vicinity increases, and it should also be positively

TABLE VI.—*Cancer of Lung and Bronchus :* Mortality in County Boroughs and London (Grouped where Boundaries are Contiguous) Arranged in Order of Total Occupied Dwellings* (1931). *Periods* 1921–39 *and* 1946–49.

County Boroughs and London.	Hundreds of occupied dwellings, 1931.	Area in acres (hundreds), 1951.	Persons per acre, 1951.	Deaths from cancer of lung, etc., in—				Comparative mortality.			
				1921–39.		1946–49.		1921–39 indices(1).		1946–49.	
				M.†	F.	M.†	F.	M.	F.	Calculated deaths(2) (males).†	C.M.R.(3) (males).
London	7306	749	45	5354	1680	3949	860	154	146	2470·9	160
Croydon	533	127	20	193	77	222	48	108	125	186·7	119
East Ham	293	33	37	133	41	127	24	117	122	88·6	143
West Ham	493	47	36	280	71	184	28	133	115	116·5	158
Area 1 total	8625	956	41	5960	1869	4482	960			2862·7	156
Birmingham	2367	511	22	1043	343	1030	217	138	148	762·7	135
Smethwick	199	25	30	92	16	90	10	148	89	57·0	158
Walsall	233	88	13	62	22	97	21	79	100	78·8	123
West Bromwich	177	72	12	52	16	62	15	88	98	57·1	109
Area 2 total	2976	696	20	1249	397	1279	263			955·6	134
Manchester	1774	273	26	1078	262	791	186	186	144	487·0	164
Salford	497	52	34	227	59	217	43	133	113	124·0	175
Stockport	328	80	18	101	13	134	23	91	36	109·4	142
Area 3 total	2599	405	25	1406	334	1142	252			720·4	159
Liverpool	1739	273	29	898	242	845	138	149	130	498·2	170
Bootle	149	31	24	73	27	77	17	130	165	39·7	194
Birkenhead	305	86	17	120	36	127	31	105	102	99·6	128
Wallasey	231	59	17	75	29	105	23	97	105	73·0	144
Area 4 total	2424	449	25	1166	334	1154	209			710·5	162
Leeds	1261	383	13	671	185	561	123	169	150	374·4	150
Bradford	831	255	11	234	76	249	71	92	89	225·3	111
Halifax	285	141	7	105	22	84	20	118	73	78·4	107
Area 5 total	2377	779	11	1010	283	894	214			678·1	132
Sheffield (*Area* 6)	1238	396	13	609	163	542	94	144	136	401·8	135
Newcastle-on-Tyne	601	111	26	187	53	262	57	83	83	217·2	121
Gateshead	266	45	26	70	13	80	15	74	49	81·0	99
Area 7 total	867	156	26	257	66	342	72			298·2	114
Bristol	836	264	17	244	101	338	81	75	93	327·8	103

Area 7 total	867	156	26	257	66	342	72						298·2	114
Bristol	836	264	17	244	101	338	81	75	93				327·8	103
Kingston-on-Hull	744	141	21	242	68	272	55	102	100				199·4	136
Nottingham	689	162	19	314	68	280	51	145	96				209·8	133
Leicester	607	170	17	114	56	208	43	56	85				208·5	100
Stoke-on-Trent	581	212	13	164	50	223	33	84	89				192·7	116
Portsmouth	546	92	25	145	67	152	32	74	106				166·6	91
Group 8 total	500–849			1223	410	1473	295						1304·8	113
Bolton	466	153	19	73	24	124	22	50	51				135·1	92
Cardiff	430	151	16	147	36	219	38	82	71				173·8	126
Coventry	413	191	14	91	33	179	38	85	94				181·0	99
Group 9 total	400–499			311	93	522	98						489·9	107
Southampton	381	92	19	94	35	133	25	64	82				131·2	101
Oldham	361	53	23	73	22	78	19	61	61				100·6	78
Swansea	348	216	4	122	34	161	22	93	96				122·1	132
Derby	343	81	17	64	22	104	21	56	66				110·4	94
Plymouth	334	131	16	97	35	107	21	57	65				146·4	73
Blackburn	327	81	14	42	15	90	13	40	43				94·5	95
Norwich	321	81	15	68	27	112	15	67	79				91·5	122
Sunderland	320	86	21	78	35	124	30	60	93				125·0	99
Huddersfield	316	141	9	101	48	94	18	98	145				106·9	88
Wolverhampton	309	91	18	93	29	129	14	92	99				112·5	115
Brighton	306	125	12	99	34	173	41	78	69				122·7	141
Preston	301	57	21	71	14	104	15	76	44				87·9	118
Group 10 total	300–399			1002	350	1409	254						1351·7	104
Middlesbrough	291	71	21	74	21	107	15	71	79				98·1	109
Southend-on-Sea	274	103	15	129	36	148	38	130	95				116·4	127
Burnley	266	47	18	62	17	50	18	72	64				72·9	69
Rochdale	255	96	9	64	23	70	28	79	89				73·0	96
South Shields	255	49	22	31	11	98	13	35	44				75·7	130
Bournemouth	243	114	13	84	45	112	33	94	110				112·8	99
Blackpool	239	85	17	67	38	105	31	72	107				132·1	80
Northampton	231	62	17	44	23	56	18	52	88				84·6	66
Reading	231	91	12	36	26	86	13	42	94				87·8	98
Ipswich	219	87	12	49	20	66	16	66	85				79·5	83
St. Helens	214	79	14	42	16	103	17	52	80				79·5	129
Grimsby	205	55	17	50	22	74	9	66	112				66·6	111
York	201	64	16	70	18	86	12	95	81				78·2	110
Group 11 total	200–299			802	316	1161	261						1157·2	100

continued overleaf

TABLE VI—cont.

County Boroughs and London.	Hundreds of occupied dwellings, 1931.	Area in acres (hundreds), 1951.	Persons per acre, 1951.	Deaths from cancer of lung, etc., in—				Comparative mortality.			
				1921–39.		1946–49.		1921–39 indices(1).		1946–49.	
				M.†	F.	M.†	F.	M.	F.	Calculated deaths(2) (males).†	C.M.R.(3) (males).
Southport	188	97	9	51	22	86	21	76	77	72·2	122
Wigan	186	51	17	38	10	56	16	56	52	63·8	88
Newport	175	74	14	60	11	90	9	78	52	76·9	117
Darlington	175	65	13	22	12	33	11	37	68	66·5	50
Warrington	173	44	18	44	16	66	10	73	101	57·0	116
Lincoln	170	61	11	27	7	41	6	46	40	55·8	73
Oxford	169	84	12	40	23	64	15	68	107	69·0	93
Barnsley	166	78	10	46	14	45	4	84	99	54·4	83
Rotherham	163	93	9	34	10	49	12	59	70	61·4	80
Merthyr-Tydfil	162	178	3	9	3	39	2	13	19	50·8	77
Bath	156	63	13	42	17	56	12	68	64	59·8	94
Doncaster	153	84	10	37	12	59	9	70	86	60·4	98
Bury	152	74	8	21	6	37	3	40	36	47·6	77
Exeter	152	90	8	40	24	41	10	73	124	57·3	72
West Hartlepool	152	42	17	19	7	40	13	35	46	60·9	66
Dewsbury	149	67	8	40	10	39	10	86	68	41·4	94
Barrow-in-Furness	148	110	6	32	12	42	4	52	79	56·1	75
Hastings	144	73	9	40	24	50	19	72	90	50·8	99
Great Yarmouth	143	36	14	34	8	23	9	70	48	39·2	59
Wakefield	142	58	10	27	12	46	4	52	89	47·3	97
Carlisle	139	61	11	13	4	46	4	30	28	48·5	95
Dudley	134	41	15	21	7	44	7	49	57	48·0	100
Tynemouth	133	47	14	34	6	35	8	66	42	47·0	74
Worcester	128	54	11	35	8	37	19	80	53	45·8	81
Gloucester	126	53	13	15	10	44	5	32	70	47·6	92
Burton-on-Trent	120	42	12	17	4	29	7	40	32	39·2	74
Eastbourne	116	110	13	30	24	48	17	64	113	44·2	109
Chester	92	41	12	16	14	44	7	48	132	36·2	122
Canterbury	60	47	6	18	8	21	7	85	107	18·6	113
Group 12 total . Less than 200				902	345	1350	280			1523·7	89

* Includes pleura.

† Excluding non-civilians except from 1931 to September, 1939, the populations corresponding.

(1) Per cent ratio of registered deaths to number calculated when mean annual rates for all County Boroughs in aggregate at ages 0–, 15–, 25–, 35–, 45–, 55–, 65–, 75+ are applied to the populations at those ages, such populations being calculated from the annual estimates at all ages and the sex-age distributions at 1931 census, the mid-point.

(2) By applying national rates at 0–, 35–, 45–, 55–, 65–, 75+ to the populations at those ages at the end of 1947.

(3) Per cent ratio of registered to calculated deaths.

correlated with the density per acre. Comparative Mortality Ratios (C.M.R.) for males in 1946–49 were as follows :

Groups of adjacent towns with over 200,000 occupied dwellings :	
London, East Ham, West Ham, Croydon	156
Birmingham, Smethwick, Walsall, West Bromwich	134
Manchester, Salford, Stockport	159
Liverpool, Bootle, Birkenhead, Wallasey	162
Leeds, Bradford, Halifax	132
Sheffield, with 124,000 occupied dwellings	135
Newcastle and Gateshead, with 87,000 occupied dwellings	114
Aggregate of 6 towns, each with 50,000 to 85,000 occupied dwellings	113
Aggregate of 3 towns, each with 40,000 to 50,000 occupied dwellings	107
Aggregate of 12 towns, each with 30,000 to 40,000 occupied dwellings	104
Aggregate of 13 towns, each with 20,000 to 30,000 occupied dwellings	100
Aggregate of 29 towns, each with less than 20,000 occupied dwellings	89

Within the groups having less than 50,000 occupied dwellings there was a large range of mortalities for individual towns, explicable by the small numbers of deaths and consequent chance variation, and by special peculiarities. For example the seaside resorts Brighton, Southend, Eastbourne and Southport had rather high rates, but they are dormitory towns and places to which many men retire after working or living in London, Manchester and Liverpool, and are therefore exceptions which would " prove the rule". St. Helens, Warrington, Cardiff and Swansea also had higher rates than their sizes appeared to warrant. There appears to be a general tendency, however, for lung cancer mortality to increase with total number of chimneys until the dwellings exceed 100,000, after which some other factor must be invoked to account for the very high rates in London, Manchester and Merseyside. That other factor could be density per acre, which is 41 in the London area, and 25 in Manchester and Merseyside, compared with 20 in the Birmingham and 11 in the Leeds group. Taking all the County Boroughs separately, there is a correlation coefficient of 0·47 between lung cancer mortality and persons per acre.

Whatever may have been the position about differing completeness of diagnosis and certification 20 years ago, they could hardly now account for such definite relations between lung cancer death rates and the extent and density of built-up areas ; nor can it be supposed that people smoke twice as much tobacco in large towns as in small. The facts, including those brought out in Tables II and III, seem to fit in with the hypothesis that atmospheric pollution by smoke is an important factor on which tobacco smoking is superimposed. The carcinogenic substance, whatever it is, might be common to both kinds of smoke, or these might contain different irritants. The findings of Waller (1952) and of Goulden, Kennaway and Urquhart (1952) on the amounts of bedzpyrene and of arsenic in the air of large towns are of great interest in this connection.

Wind direction as a possible factor.

Returning now to Greater London, Clark (1951) has shown that the logarithm of the population density per acre in successive zones diminishes in arithmetic progression on passing outwards from the business centre, and the same is true of other large cities. Supposing domestic smoke to be an important factor in producing lung cancer, if there was no wind at all one would expect to find the highest incidence at the centre, diminishing in all directions outwards. If there is wind and it comes from all directions equally, a more diffused but still symmetrical distribution would be expected, with less contrast near the centre and more near the periphery. However, wind does not blow equally from all directions, and Greenwich records over 12-hour periods throughout the year 1950 show that the wind direction, when analysed into 8 sectors, was S.W. for 35 per cent of the time, W. for 15 per cent, S. for 14 per cent, N.E. for 10 per cent, N.W. for 8 per cent, E. for 7 per cent, N. for 6 per cent, and S.E. for 5 per cent. During half the time, therefore, wind came from one quarter of the circle distributed around W.S.W., whereas from the reverse quarter distributed around E.N.E. the wind blew for only one sixth of the time. The expected effect of that on smoke density would be to shift the maximum some distance from the centre of Greater London in a direction E.N.E., and that is what the map for lung cancer mortality in London County shows, the black patch covering Finsbury, Shoreditch, Bethnal Green, Stepney and Bermondsey. There is also a positive correlation of 0·37 with density per acre in the Metropolitan boroughs, which are of course affected by their own smoke as well as by that blown from other parts of London.

Atmospheric pollution and tobacco smoking.

How could this hypothesis be squared with the Medical Research Council figures about tobacco smoking in Greater London ? If 90 per cent of lung cancer in that area was due to tobacco alone, it could not be squared ; but, remembering that about one-third of the female patients with lung cancer had not smoked, the figures are not incompatible if it be supposed that the effects of tobacco and atmospheric pollution are additive. This is easier to perceive in terms of an analogy. Suppose there are set out in a garden 50 jars with capacities ranging from 5 to 10 pints, one-fifth each holding 6, 7, 8 and 9 pints, and one-tenth each holding 5 to 10 pints. Arrange them in 5 rows of 10 each, evenly distributed as to capacity, and suppose that water does not evaporate. During a year leave the first row alone, add 1 pint of water to each jar in the 2nd row, 2 pints to the 3rd row, 3 pints to the 4th row, and 4 pints to the 5th row, and count how many jars have overflowed in each row during the year. Suppose one finds 1 in the first row, 3 in the 2nd, 5 in the 3rd, 7 in the 4th, and 9 in the 5th. If one did not know the capacities of the jars and forgot that it had rained a lot during the year, it would appear that overflow depended entirely on the water added, except that 1 out of 10 overflowed without doing anything. But in actual fact each of the 25 jars which overflowed received 5 pints of rain water, a total of 125 pints, and there were added only 70 pints, so rain was really a bigger factor in producing the overflow than was the water added.

Like all analogies, this will break down if pushed too far, but it shows that the total contribution of tobacco smoking to lung cancer incidence cannot be deduced

from the relative frequencies found amongst non-smokers and smokers of 10, 20, 30, 40 cigarettes a day in Greater London alone. It will be necessary to do similar studies amongst residents of rural areas, where it is conceivable that smoking does not begin to show an appreciable relation with incidence of lung cancer until the number of daily cigarettes is at a higher level than appears to be the case in London. In terms of the water jar analogy, if the experiment is repeated where the rainfall is smaller and contributes only 3 pints a year instead of 5, overflow would only begin at the 3rd row and then increase. This would mean, in effect, that smoking is more dangerous to a town dweller than to a country dweller.

This hypothesis is no more than a theory which seems to fit most of the statistical findings so far available. It has not been proved, and may be found later to be untenable. It can be tested, however, and plans are now ready, thanks to the British Empire Cancer Campaign, and its Cheshire and North Wales Group, to collect data during the next five years throughout North Wales and Liverpool Hospital Region in the hope of elucidating this hypothesis about lung cancer at the same time as other field studies are being made concerning pathogenesis of cancer of the stomach and other organs. In that work the hospitals, radium institutes and medical officers of health are co-operating.

Although, as one is continually reminded, statistics in themselves cannot establish causation, and in common with other types of cancer research they often lead up blind alleys, they can start what biochemists and pathologists will finish, and they may even succeed in making sense out of a chaos of isolated findings by other research workers. It seems possible that this may happen sooner for lung carcinoma than for any other form of cancer.

In conclusion, I have to acknowledge my indebtedness to the Registrar-General for access to unpublished details of deaths in 1931–46, and to former colleagues at the General Register Office who helped in their extraction.

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ONE HUNDRED CONSECUTIVE PRIMARY EPITHELIAL LUNG TUMOURS.

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In many countries the statistical reports show a steady and remarkable increase in the number of deaths from "lung cancer." The problem puzzles the cancer specialists and troubles the public and the medical profession. Does this increase signify a real augmentation of cases of lung cancer, or does it simply mean an increasingly better diagnostic service? This question will not be discussed in this paper.

If we wish, however, to study, by statistical methods, the possible role of certain irritants in the genesis of lung cancer we have to make clear what we mean by the term "lung cancer."

According to Kennaway and Kennaway (1947) their statistics from England and Wales comprise: "Cancer, carcinoma, or sarcoma of lung, bronchus, pleura, root of lung, hilum of lung, lung and mediastinum, or lung and pleura. Pulmonary, or bronchial carcinoma or sarcoma. Cancerous pleurisy."

The official Norwegian mortality statistics, up to the publication of the latest figures, have followed a similar pattern, with minor changes during two different periods (Fig. 1).

It, is, however, unlikely that all the morbid conditions included are caused by or influenced by the same factors. Primary carcinoma of pleura is a very rare condition and would not disturb our statistics significantly. Malignant diseases of the hilum of the lung, on the other hand, may include malignant lymphomas, reticulosarcomas, Hodgkin's disease and other malignant or semi-malignant diseases. In the group "lung cancer" may also be included a number of neurinomas and even an unknown number of infectious or non-infectious lung diseases.

It is therefore with great satisfaction we acknowledge the fact that the World Health Organisation has prepared a new classification, according to more precise diagnoses. The Norwegian "Statistisk Sentralbyrå" has, since the first of January, 1951, acted accordingly. It is a great step forward.

In order to study a possible relationship between lung carcinoma and smoking habits, or industrial irritants, it may be of importance, not only to exclude all other lung tumours than primary lung carcinomas, but even to subdivide the latter into special sub-groups.

From our general experience in the field of cancer research we are ever more impressed by the importance of the study of the geographical distribution of the different manifestations of malignant disease. This different geographical distribution examined on the background of the corresponding difference as to races,

habits and social conditions may be an important means in uncovering some of the secrets of the development of cancer.

For these reasons we have embarked upon a long term project in mapping the occurrence of lung carcinomas in Norway, with the first main object of examining the types of lung tumours most prevalent and next of examining the smoking habits and the occupational conditions of those afflicted by true epithelial lung tumours. This report deals with 100 consecutive cases of primary epithelial lung tumours, received for diagnosis at the Institute of Pathology, at the University of Oslo. All, but also only those, tumours are included, where the piece of tissue

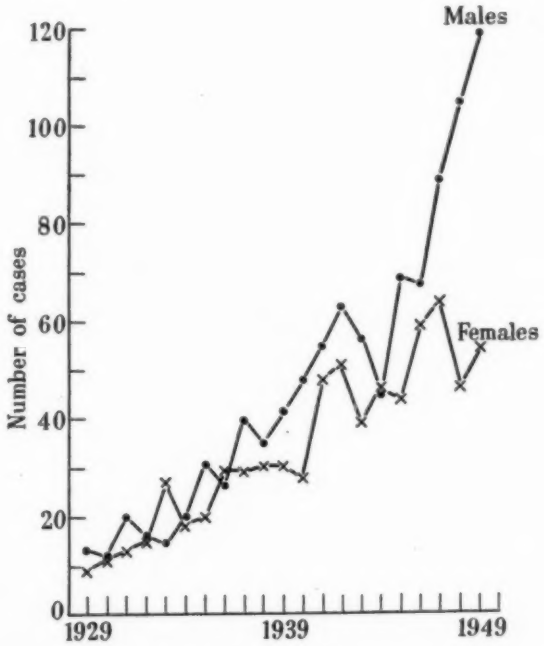


FIG. 1.—Mortality statistics from Norway, 1929 to 1949 (" Statistisk Sentralbyrå ").

was large enough to permit a definite histological classification. The main part of the material (97 cases) was received from the Surgical Department A, at the University Hospital, 2 cases came from the Ear, Nose and Throat Department, and 1 case from the Roentgen-Radium Department. Hereto are added 5 cases of secondary lung tumours, received within the same period, and clinically regarded as primary tumours, the histological examination, however, revealing their true character (2 cases of metastasizing thyroid adenoma, and 1 case each of hypernephroma, adenocarcinoma of uterus and malignant naevus tumour).

The 100 tumours of the main series were classified according to traditional terminology as follows :

A. *Adenocarcinoma* has been the designation when a clear-cut atypical, infiltrating cylindrical epithelium is present, with or without mucus secretion. In a few cases special adenocarcinomas have been grouped, as stated below.

B. *Squamous cell carcinoma* includes tumours with a more or less marked stratification or whorl formation of the tumour cells, or when keratosis or parakeratosis is present, or epithelial bridges can be seen. In a small, but not insignificant, number of cases pictures may be found presenting adenocarcinoma in one area and squamous cell carcinoma in another. These cases have been placed in an intermediate group (AB). Every experienced pathologist will agree that borderline cases exist, and that, accordingly, subjective factors may explain a different classification even of the same material. In the present group controversy may especially arise as to the proper classification of squamous cell carcinoma, " highly atypical," on the one hand, and the following group, on the other.

C. *Large cell carcinoma*.—This group comprises tumours with large atypical cells, irregular growth and no differentiation.

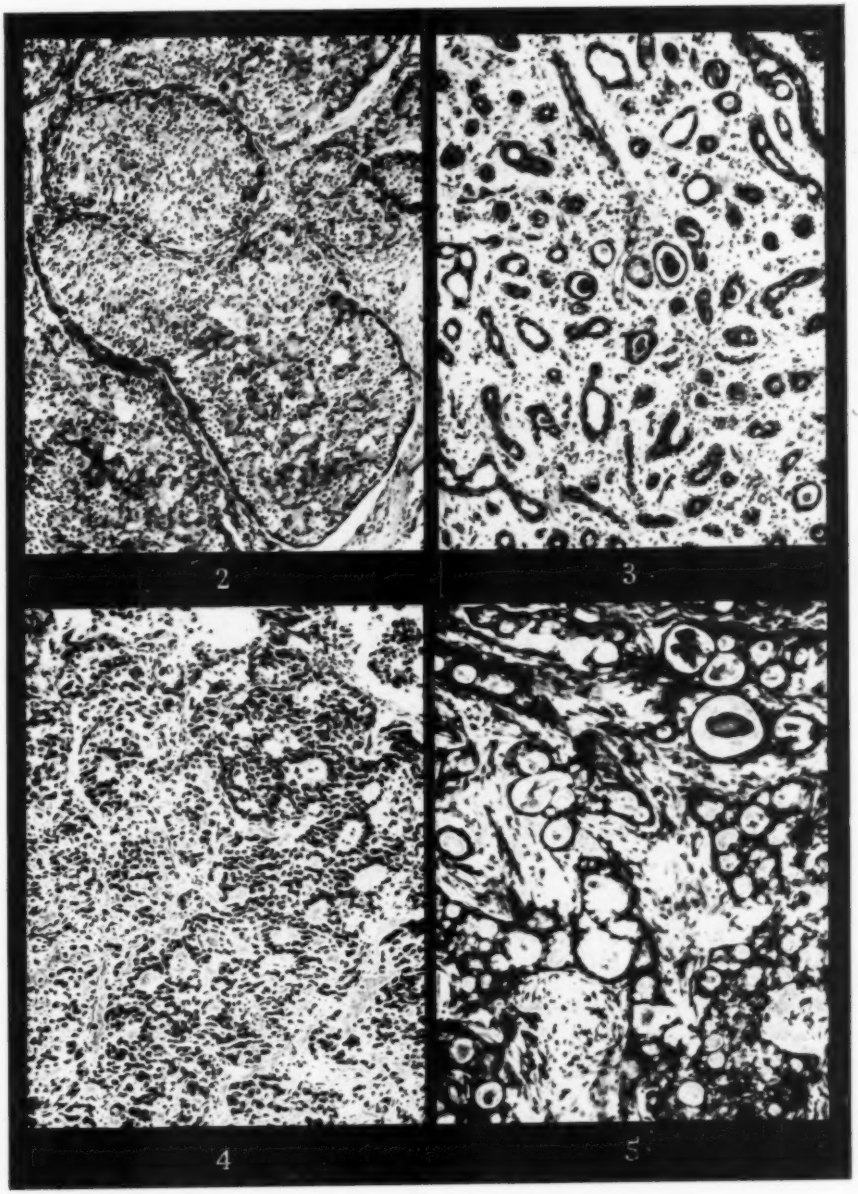
D. *Small cell carcinoma* (" oat-cell " tumour) represents an important group with a characteristic microscopic picture, which, usually, does not offer great diagnostic difficulties if the material at hand is sufficient in size and properly fixed and stained. In small pieces and in smears, or in badly fixed material,

TABLE I.—*Age Distribution of the Various Groups of Lung Tumours.*

Type.		Age-groups. 11–20.	21–30.	31–40.	41–50.	51–60.	61–70.	71—.	Number of tumours.	Total.
A. Adeno-carcinoma	♂	..	..	..	2	2	..	..	4	7
	♀	..	..	..	3	..	..	..	3	
B. Squamous cell carcinoma	♂	..	..	3	9	21	7	2	42	45
	♀	..	..	..	1	2	..	..	3	
AB. Mixed A and B	♂	..	..	..	1	..	..	..	1	4
	♀	..	..	..	1	1	1	..	3	
C. Large cell carcinoma	♂	..	..	..	1	2	3	..	6	8
	♀	..	..	..	1	1	..	..	2	
D. Small cell carcinoma	♂	..	..	1	1	7	5	..	14	17
	♀	..	..	..	1	2	..	..	3	
E. Papillary × alveolar carcinoma	♂	..	1	..	..	1	..	..	2	3
	♀	..	..	..	..	1	..	..	1	
F. Salivary gland tumours	♂	..	1	1	2	4	1	..	9	16
G. Adenomas	♀	1	1	2	1	..	2	..	7	

EXPLANATION OF PLATES.

FIG. 2.—Salivary gland adenoma, Pp 1043/48, in a 69-year-old woman (Table II). × 100.
FIG. 3.—Cylindroma, Pp 1945/49, in a 63-year-old male (Table II). × 100.
FIG. 4.—Muciparous salivary gland carcinoma, Pp 1281/49, in a 38-year-old male (Table II). × 100
FIG. 5.—Salivary gland carcinoma with cylindromatous areas, Pp 1487/48, in a 38-year-old woman (Table II). × 100.
FIG. 6.—" Oat-cell " carcinoma, Pp 1197/48, in a 56-year-old male. × 400.
FIG. 7.—Malignant adenoma, Pp 2343/50, in a 23-year-old woman (Table II). × 400.
FIG. 8.—Benign " trabecular " adenoma, Pp 1793/46, in a 61-year-old woman (Table II). × 200.
FIG. 9.—Benign " carcinoid " adenoma, Pp 2061/49, in a 51-year-old male (Table II). × 200.



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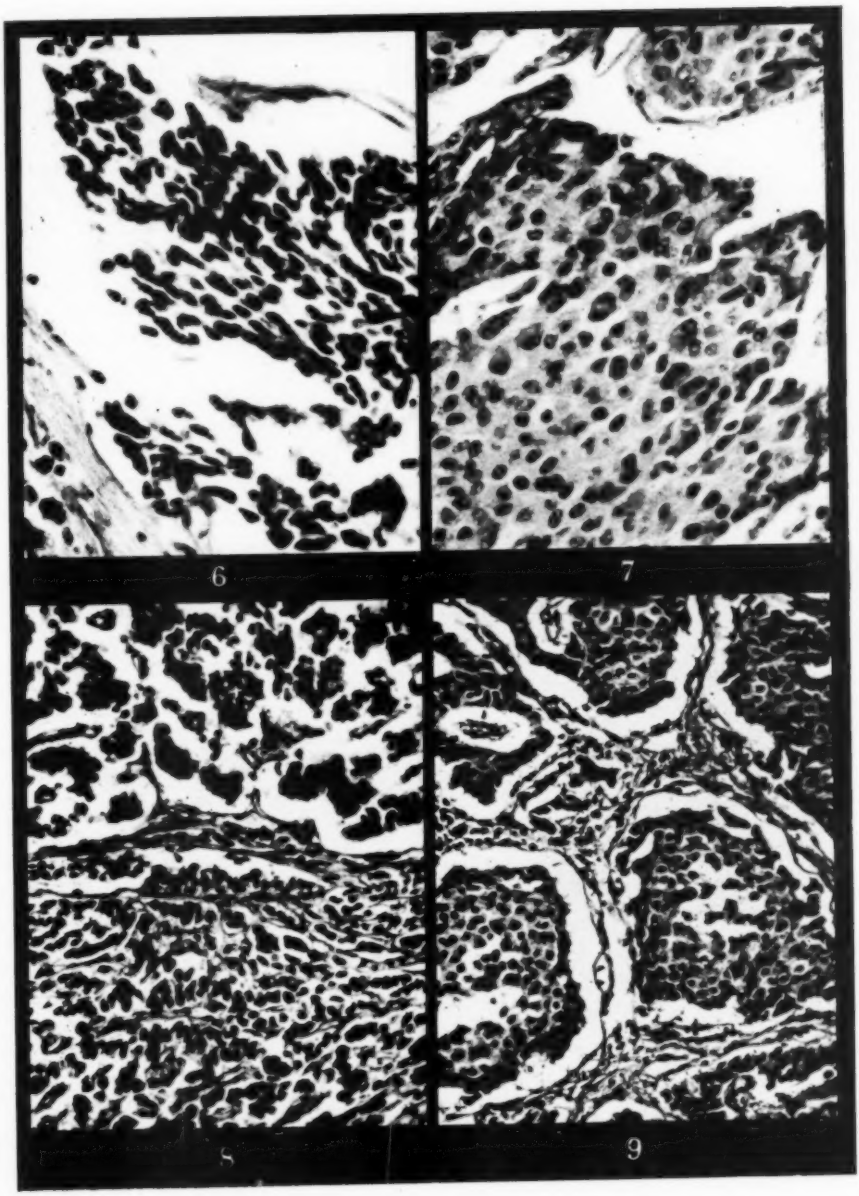

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certain differential diagnostic difficulties may arise *vis-à-vis* a malignant adenoma (Fig. 6, 7).

E. *Papillary and alveolar carcinomas* form a small but characteristic group of adenocarcinomas, the peculiar picture usually making the diagnosis easy.

F. *Salivary gland tumours*, benign, malignant and semimalignant, are not at all rare, in our material, represenitng 5 out of 100 tumours. The same types are encountered as found in the parotid region, the mixed tumours, however, being rare in the lung. In some of the malignant tumours a definite adenocarcinomatous picture may be found, sometimes with a mucous secretion, but usually the pattern of the salivary gland tumours may still be recognized (Fig. 2, 3, 4, 5).

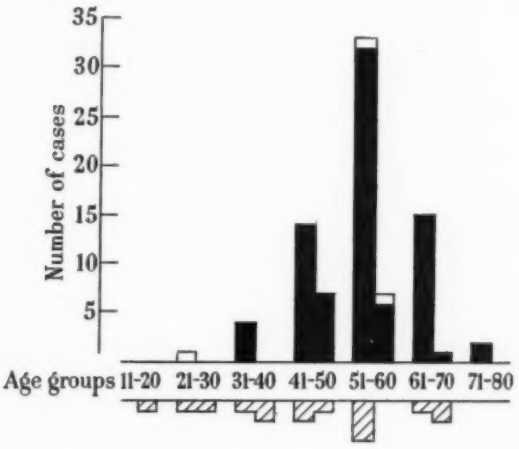

FIG. 10.—The distribution in age-groups of the 100 primary epithelial lung tumours. Males in the first column, females in the second.

■ True bronchogenic carcinomas.

▨ Adenomas and salivary gland tumours.

□ Papillary and alveolar carcinomas.

G. *Adenomas* are in most cases easily distinguishable by their trabecular or carcinoid pattern (Fig. 8, 9), and with intermediate forms. In our series 11 occurred. Not a few develop towards malignancy, with metastases in the lymph nodes (Fig. 7).

From Table I and Fig. 10 the number and the different types of tumours of our main series may be seen, as well as the occurrence in age-groups and the distribution between the two sexes. The individual tumours of Groups G and F are tabulated in Table II.

The Groups E, F and G do not show any marked preponderance in the one sex or the other. Further, these tumours are rather evenly distributed in the different age-groups, and they occur even in very young people, our youngest patient being a girl of 19 with a malignant adenoma. The papillary and alveolar carcinomas may have their specific, yet unknown, etiology. The salivary gland

TABLE II.—*Age Distribution of the Salivary Gland Tumours and Adenomas.*

Tumour.	Age-groups.					
	11–20.	21–30.	31–40.	41–50.	51–60.	61–7[illegible].
F. Salivary gland tumours and G. Adenoma	Malignant adenoma, ♀ (1569/47)	Malignant adenoma, ♀ (2343/50)	Malignant adenoma, ♀ (422/50)	"Trabecular" adenoma, ♂ (120/50)	"Carcinoid" adenoma, ♂ (2061/49)	"Trabecular" adenoma, ♀ (1793/46)
	..	Muciparous adenocarcinoma from salivary gland tumour, ♂ (1807/50)	Muciparous carcinoma from salivary gland tumour, ♂ (1281/49)	"Trabecular" adenoma, ♂ (1183/51)	"Trabecular". small alveolar ♂ (378/51)	Salivary gland adenoma, ♀ (1043/48)
				"Trabecular" semimalignant adenoma, ♀ (953/48)	"Trabecular" and "carcinoid" adenoma, ♂ (1267/49)	Cylindroma, ♂ (1945/49)
			Salivary gland carcinoma, ♀ (1487/48)		Malignant adenoma, ♂ (819/49)	

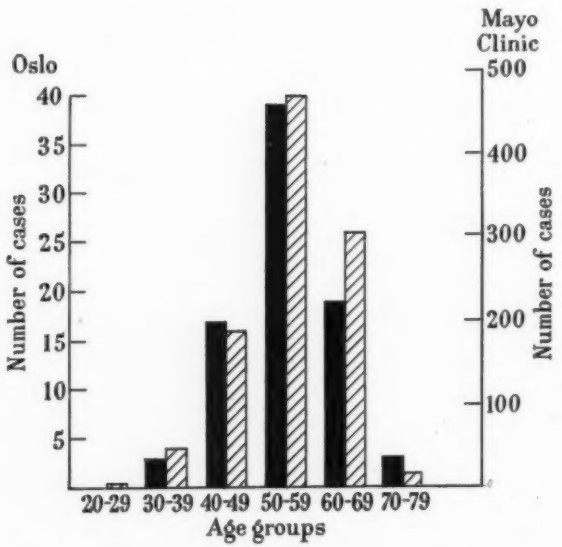

FIG. 11.—The age-distribution of the true bronchogenic tumours in the Oslo group and the material from the Mayo Clinic.
■ Oslo material.
/// Mayo Clinic material.

umours and the adenomas most probably originate from developmental irregularities.

The Groups A, B, C and D in our material, in complete accord with general experience, show a marked preponderance of male sufferers, most marked in the small cell carcinoma and least in the adenocarcinoma. These types of cancer are very rare before the age of 40, and they often show a very marked peak in the age-group between 50 and 60. These are the tumours often designated *bronchogenic carcinomas.*

The findings in the Groups A, B, C and D, comprising 81 tumours, have been compared to a material, ten times as large from the Mayo Clinic, recently presented in four different papers by Patton, McDonald and Moersch (1951*a*), Carlisle, McDonald and Harrington (1951), Patton, McDonald and Moersch (1951*b*) and McBurney, McDonald and Clagett (1951). After having reduced the Mayo Clinic figures to the Oslo figure scale, the two materials are compared as to age distribution. The Mayo Clinic material comprises the same types of lung carcinomas as our Groups A, B, C and D. From Fig. 11, it will be seen that the two groups are practically identical as to age distribution.

TABLE III.—*Comparison of the Oslo and Mayo Clinic Material.*

Type.	Oslo.		Mayo Clinic.	
	Number.	Per cent.	Number.	Per cent.
A. Adenocarcinoma	7	9	112	13·2
B. Squamous cell carcinoma	45	56	321	37·8
AB. Mixed A and B	4	5	..	..
C. Large cell carcinoma	8	10	314	40·7
D. Small cell carcinoma	17	21	75	8·8
Total	81	..	822	..

Quite another picture will be found when we examine the occurrence of the different tumour types (Table III). I do not at all, *a priori*, accept this as a token of heterogenity of the two materials, referring to my remarks above, as to the strong individual factors involved in the act of classification. The homogenity as to tumour occurrence in age-groups seems to me more significant. Both materials were of surgical origin.

The figures from a recent Swedish report upon a surgical material of bronchogenic carcinoma (Wiklund, 1951) are rather similar. The distribution as to age-groups is nearly identical with the figures from the Mayo Clinic and Oslo. The sex difference is, however, still more pronounced, Dr. Wiklund having 91·1 per cent males in his series. As to the microscopical classfication, he has : adenocarcinomas 4·6 per cent, squamous cell carcinoma 62·9 per cent, undifferentiated carcinoma 25·1 per cent and uncertain type 7·4 per cent. Here again the similarity is greater when we examine the material on the basis of the more objective characteristic the distribution in age-groups than on the basis of the more subjectively influenced classification in histological types.

It is evident that the surgical material cannot, without a closer examination, be taken as representative for the occurrence and the distribution of lung carcinomas and lung tumours in the population. Firstly, cases occurring in old age

will not be represented, as these patients are poor operative risks and seldom operated upon. Secondly, in old age the diagnosis may be more difficult and possibly not pursued with the same enthusiasm as when the patient is a younger. Thirdly, the sex distribution may be different. It is not unlikely that the local doctor, the surgeon and the patient himself will take a greater risk and show a somewhat more heroic attitude towards the question of operation when the patient is a male. The women in these age-groups may possibly show more resignation to the blows of fate. Fourthly, surgical material may show a greater number of benign and moderately malignant tumour types than the sum total. This is significant when the material examined includes all primary epithelial lung tumours, but less important when only true bronchogenic carcinomas are concerned. This underlines the importance of a careful classification when statistics are to be compared.

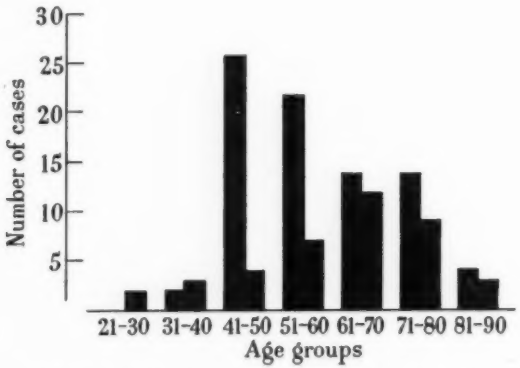

FIG. 12.—The age distribution of the cases of lung cancer from the post-mortem material at Ullevål Hospital (Dr. Arnt Jakobsen, personal communication). Males in the first columns, females in the second, for each age-group.

The relevance of these remarks is partly confirmed through a study of a rather representative post-mortem material from Oslo City Hospital (Ullevål Hospital). The figures have been placed at my disposal through the kindness and courtesy of Dr. Arnt Jakobsen in a personal communication. The material comprises 122 cases (82 males and 40 women) of primary " lung cancers " histologically verified, but not histologically classified, during the period (1937 to 1946) (Fig. 12).

The sex ratio in this autopsy material is male : female, 2 : 1, which is considerably closer to the sex ratio in our general mortality statistics (also very near 2 : 1 up to the last 2 years) than in my surgical material, where the ratio was 4 : 1 (78 : 22) in the whole group, and nearly 5 : 1 (67 : 14) in the sub-group of pure bronchogenic carcinomas. As was anticipated, a considerable number of cases were found in the higher age-groups. The occurrence of a certain number of cases in the early age-groups indicates that a few adenomas are included also in this post-mortem series.

I wish to emphasize that the present paper represents a preliminary orientation only, as to the occurrence of lung carcinomas in Norway. The figures may,

however, be of a certain interest. Firstly, the different statistics confirm the general impression that in Norway the preponderance of the male sex in the lung cancer statistics is not as marked as in many other countries. Secondly, the occurrence of lung tumours in surgical material in different countries may represent fairly comparable material, but they differ widely from the actual occurrence in the population total and as it may be found in autopsy material Thirdly, up to the present time the general mortality statistics are so unprecise that they must be used with great reservation as a base for the study of the actual occurrence of lung cancer. In the present clinically select material of 105 cases of " clinically " primary lung cancers 5 were shown to be secondary, and in 19 cases the tumours belong to types where external irritants most probably do not play any role at all.

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NEOPLASTIC DISEASE OCCURRING IN VARICOSE ULCERS OR ECZEMA: A REPORT OF SIX CASES.

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FROM perusal of the literature available it appears that chronic ulceration associated with varicose veins is the most rare cause of malignant disease of the skin of the lower limb. We therefore wish to present 6 cases which undoubtedly occurred in conjunction with a varicose ulcer or eczema which had been present for many years.

Cutaneous neoplasia of the leg is rare, and study of the literature demonstrates that such skin cancers as do occur very rarely arise *de novo*, but usually in connection with chronic inflammation following injury, in old scars and sinuses, or after some chronic skin infections such as lupus vulgaris as well as in the varicose conditions mentioned. Since the latter are without question the most commonly found benign lesions of the leg the excessive rarity of malignant degeneration is surprising and interesting.

In recent years papers on this subject have been few. Knox (1925), in an exhaustive study of the literature, collected 59 cases of epithelioma arising in varicose ulcers, and added 2 more from her own experience. She maintains that such neoplasms, whilst remaining definitely malignant growths, run a more indolent course than similar tumours elsewhere, metastasising later, and probably do not arise until the ulcer or eczema has been present for at least 15 years. She agrees with Gotheil (1912) in believing that the chronic dermatitis, rather than ulceration, which accompanies varicose veins should be regarded as the more important lesion aetiologically. In 1926 D'Assis (1926) collected 17 cases of leg epithelioma, only one of which arose on varicose ulceration, and Tenopyr and Silverman (1932), in a paper recording 19 cases of malignant cutaneous tumours of the leg, describe 4 cases as definitely occurring on old-standing varicose ulcer. This number represented an incidence of 0·4 per cent of lower leg ulcers, a figure which corresponds with figures published by Gothiel (1912), D'Assis (1926), Volkmann (1939) and others. Lang (1932) and Rubenfeld (1934) each reported one case, that of Lang giving a history of only 2 years' duration, which leads us to believe that this ulcer was malignant from its inception. Monder (1938) reports 2 cases, one of which occurred in conjunction with varicose ulcer.

Of sarcomatous degeneration we can find only three previous reports. Prewitt (1884) reported a case of fibrosarcoma which presented as a large fungating growth implanted upon a varicose ulcer of 15 years' duration. Donati (1935) reported a tumour described histologically as a "round-celled" sarcoma. This may well have been a poorly differentiated epithelioma, and Glasser (1939) reported a case in which a fibrosarcoma appeared as a large fungating growth on a long-standing varicose ulcer.

Four of our present series presented the histological features of squamous epithelioma, one was a fibrosarcoma, and one was considered to be a bone-forming mesenchymal tumour of malignant nature and was called an ostoegenic sarcoma.

Case Reports.

Mrs. E. L—, aged 83 years, had suffered from a varicose ulcer of the left leg for over 50 years. She was admitted to hospital with a huge ulcer, from the centre of which a mass of soft granulation-like tissue was growing (Fig. 1). On examination she was a wasted, frail old woman not well orientated. There were no large nodes in the groin, but several small soft nodes could be palpated. The general examination showed senile changes but no obvious disease. Biopsy of the tumour revealed a well-differentiated squamous carcinoma with cell-nests. X ray of the leg showed extensive destruction of the tibial shaft just below the mid-point with considerable soft tissue invasion. No X ray evidence of malignant disease was found in lungs or other bones.

Treatment by radium applicator was given, and after 63 hours' treatment the tumour had all but disappeared, leaving a large, flat ulcer. The general condition of the patient was much improved by her stay in hospital, but the ulcer remained unhealed and became painful. This prevented her from getting about her house and she became practically bedridden. In these circumstances there seemed no need to retain a painful limb and amputation was advised. However, during the 2 or 3 weeks this elderly lady was awaiting admission she suffered an intracranial thrombosis and died.

Mr. E. B—, aged 71 years, was referred to us for treatment of a very large varicose ulcer of 20 years' duration. Four months before his admission to hospital the lower edge had begun to hypertrophy and the ulcer to spread on to the dorsum of the foot. On examination a huge L shaped ulcer on the inner aspect of the left leg was found. The ulcer, 16 cm. long by 8 cm. wide, extended below the internal malleolus and on to the dorsum of the left foot. The base of the ulcer was yellowish slough, the edges, especially the lower edge, were heaped-up hard and surrounded by a zone of induration (Fig. 2). Varicose pigmentation and very large veins with incompetent valves were also found on both legs. The right leg had also been ulcerated, but this was healed at the time of admission. The superficial and deep inguinal nodes were palpable in both groins—they were hard, not tender, and mobile on both sides, but were larger on the left side. The Wassermann reaction was negative.

Because of the sudden increase in size and the suspicious appearance of the lower edge biopsy was performed of this edge of the ulcer. The section showed a well-differentiated squamous carcinoma with mitoses frequent (about 3 per cent) and resting cells not more than 15 per cent.

Treatment of the ulcer was begun using deep X ray therapy (190 kv.) by two fields. Progress was controlled by serial biopsy as treatment proceeded, and biopsy, after a total dose, measured at 1 cm. from the edge of the fields, of 5344 r, revealed no evidence of malignancy and a normal capillary arrangement of the epithelium. Following this treatment the patient was left with a large painful ulcer, now regarded as benign (Fig. 3). For some months he was treated by supporting dressings of elastoplast or Unna's paste and ichthyol cream, but the ulcer extended further and the pain increased. He was advised to have an above-knee amputation (since the ulcer was extending so far up the leg as to leave an inadequate below-knee stump), but this he resolutely refused, and a below-knee amputation was performed in order to relieve his pain and discomfort. This cleared the upper edge of the ulcer by only 3 cm. Pre- and postoperative chemotherapy (soluble penicillin) was given to minimise the infection and his convalescence was rapid. Healing of the skin-flaps was slow but complete. Several blocks were taken from the ulcer after amputation, but serial section revealed no sign of malignancy in any part of it. All further treatment to his enlarged groin nodes, either surgical or by

radiotherapy, was refused. Despite this he has survived to date, a total of over 5 years. An artificial limb has been fitted and he walks well. The groin nodes have not altered during this time.

Mrs. M. W—, a widow of 68 years, had a small, painful, deep ulcer of the left leg which had not responded to treatment. She had suffered from varicose eczema and ulcers for many years, she could not remember when the condition first appeared. This ulcer was deeper and more painful than any other she could remember (Fig. 4).

On examination she showed a deep ulcer over the left shin, about 2 cm. in diameter. The ulcer showed a clean base and only slight heaping of the edge There were no nodes palpable in the groin. The blood Wasserman reaction was negative. Biopsy of the ulcer edge revealed a fairly well differentiated squamous cancer showing attempts at keratinization.

Treatment by radium applicator was applied, a total of 32 g. being applied for 117½ hours. This treatment produced healing of the ulcer within 5 weeks and she was discharged home. Four months later the patient sustained a fracture of her femoral neck. X rays showed that this was not due to a secondary growth at the site of fracture. This fracture was treated by fixation of the hip in a plaster spica. Sound union was not obtained, and she died one year later of hypostatic pneumonia and senility and with her fracture unhealed. There were no signs of malignant deposits, and the area where the ulcer had been remained soundly healed to the end.

Mrs. E. H—, a widow of 67 years, was referred to us for treatment to secondary neoplastic nodes in the right inguinal region. She had been treated by mid-thigh amputation, at another hospital, for a 30-year-old decubitus ulcer which, at the time of amputation, extended from the dorsum of the right foot to one-third of the distance up the tibial shaft and involved the bone. There had been no recent change in the ulcer prior to amputation but it had steadily increased in size during about 7 years before admission to hospital. Amputation had been advised for pain, and because of the large size and failure to heal with support and dressings.

On examination her right leg had been removed through the thigh. The skin flaps were nearly healed. Hard mobile nodes were present in both groins and in the right axilla. The left leg showed marked varicose veins and the scar of a healed ulcer above the left internal malleolus.

Her heart was fibrillating (she had been receiving treatment for this for about 6 years), but there were no signs of cardiac failure.

A report on sections taken from the ulcer after amputation showed a well-differentiated keratinising squamous-celled carcinoma.

A biopsy excision of nodes palpable in the axilla was performed. No malignant

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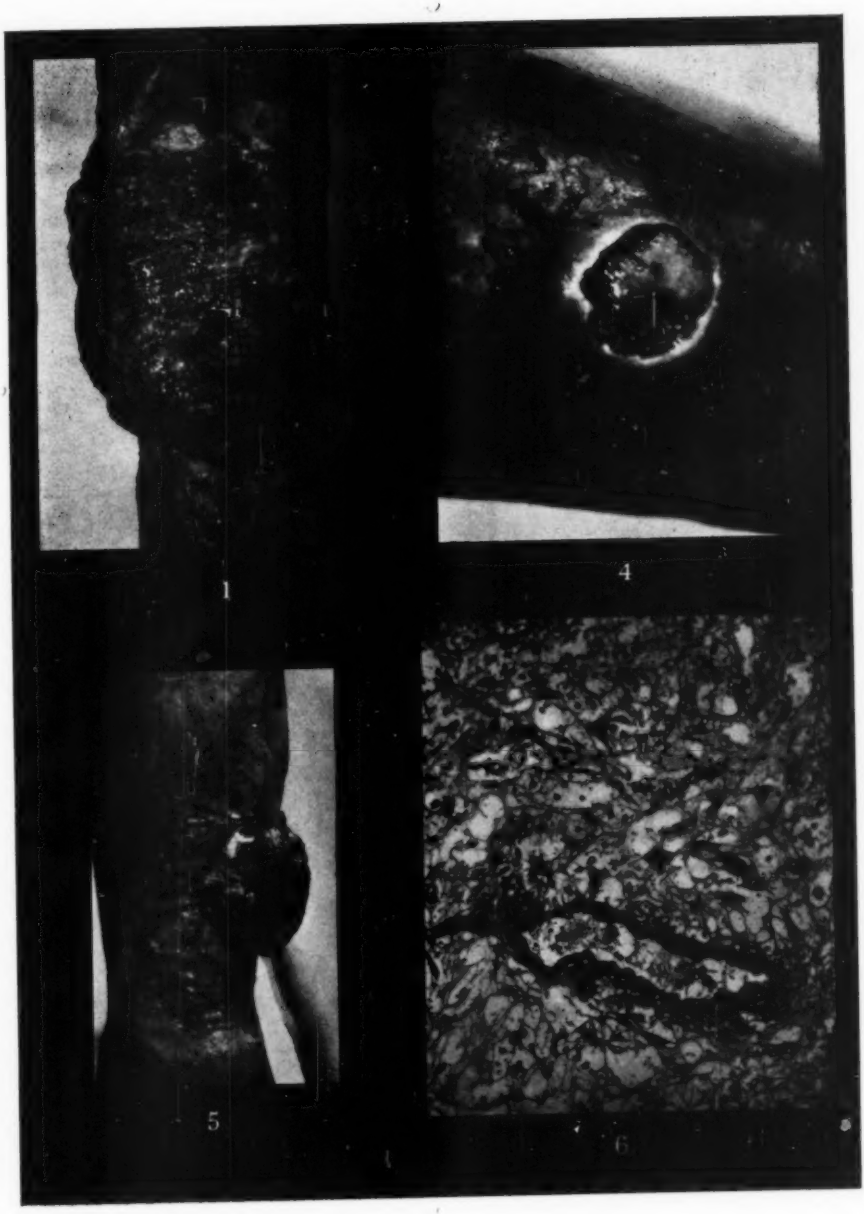
EXPLANATION OF PLATES.

FIG. 1.—Photograph of the ulcer on Mrs. E. L—'s L. leg on admission. Biopsy was performed at the upper edge, which felt harder than surrounding parts.
FIG. 2.—Photograph of the ulcer on Mr. E. B—'s leg on admission.
FIG. 3.—Leg of Mr. E. B— after completion of radiotherapy.
FIG. 4.—Photograph of ulcer on Mrs. N. W—'s leg.
FIG. 5.—Photographs of ulcer on Mr. H. D—'s leg showing fungating cauliflower tumour.
FIG. 6.—Microphotograph of the biopsy of the tumour on Mr. H. D—'s leg. × 120.
FIG. 7.—Photograph of ulcer of Mr. J. R—. The tumorous growth can be plainly seen.
FIG. 8.—Radiograph of leg of Mr. J R—, prior to treatment, showing grows calcification in the body of the tumour with no apparent connection with the underlying bone, which shows only periostitis.
FIG. 9.—Microphotograph of original biopsy of ulcer on Mr. J. R—'s leg. The squamous epithelium of skin at the ulcer's edge may be seen. × 115.
FIG. 10.—High-power microphotograph of an area of the lymphatic secondary. × 120.



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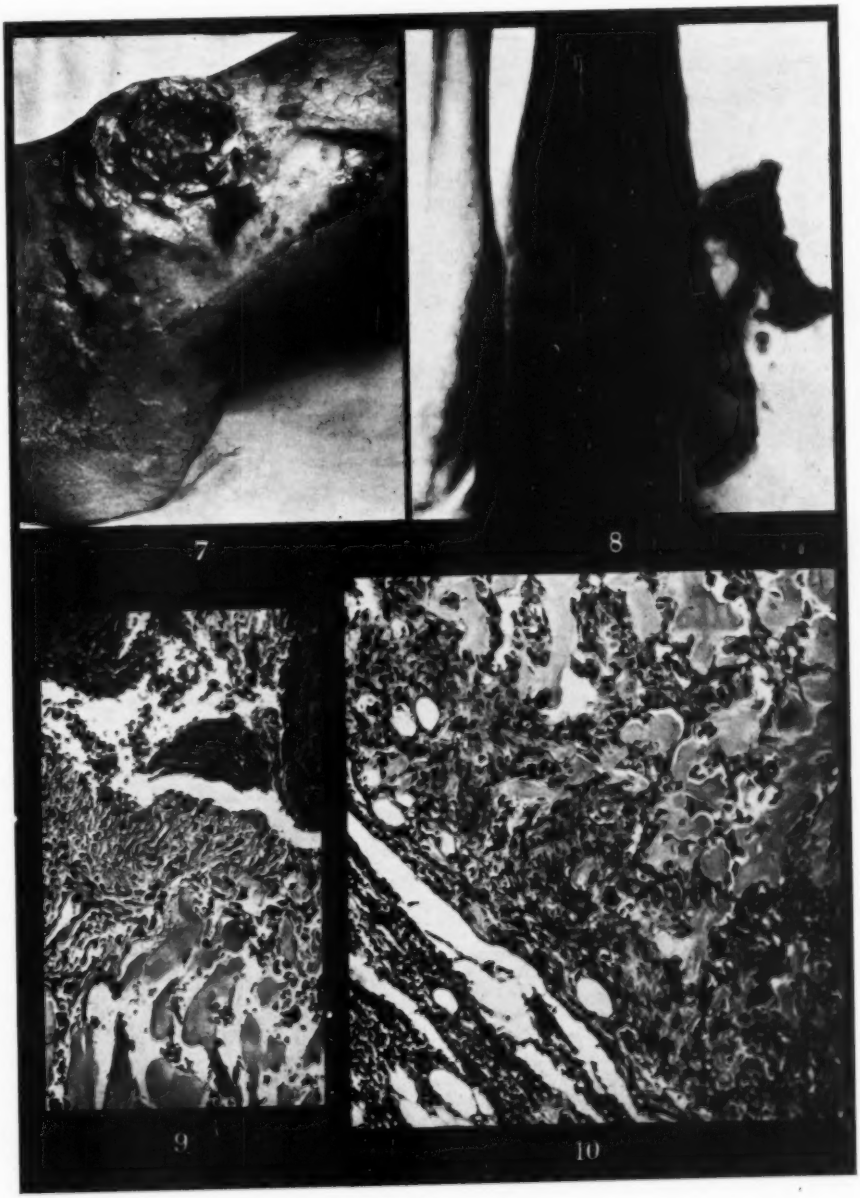

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deposit could be found in these nodes. Biopsy of the nodes palpable in the right groin revealed a tumour similar in histology to the primary.

No further metastases were revealed by X ray of the spine, pelvis or lungs. Blood count was normal. A blood Wassermann reaction was not done.

Because of her general, and in particular, her cardiac condition extensive ablation of both groups of inguinal nodes was considered inadvisable. She was therefore submitted to deep X ray therapy (220 kv.) and a total skin dose of 4500 r given to each groin.

The groins, after showing moist desquamation, cleared quickly and the patient remains alive and well to date. This represents a survival time of 6 years.

Mr. H. D—, aged 58 years, was referred for treatment of an ulcer of the right leg. He had suffered from an ulcer at this site for 20 years, during which time it had never healed completely. Seven weeks before admission a swelling had developed in the upper part of the ulcer and had grown rapidly. This tumour had bled once before his admission (Fig. 5).

On examination there was a large ulcer of the right lower leg ; in the upper part of this ulcer there was a large fungating mass covered with a dark eschar. This mass was soft, friable and necrotic, about 2·5 in. in diameter. The tumour bled freely when touched. The right inguinal nodes were enlarged, discrete, not adherent to surrounding tissues and not tender. The left nodes were also enlarged and mobile.

His general condition was poor, by reason of hypotension and chronic nephritis. His blood urea was 208 mg., W.B.C. 18,000 per cmm., 89 per cent neutrophils and Wasserman reaction negative.

X ray of the right lower leg showed gross sclerosis of the tibial shaft, and an area of necrosis of the shaft subjacent to a large soft tissue swelling. No other radiological evidence of malignancy was found.

Biopsy of the tumour showed a general structure like granulation tissue, with well-formed capillaries and abundant reticulin. Cytologically it was definitely sarcomatous, with large, pleomorphic, usually spindle-shaped cells with large irregular often multiple nuclei and numerous mitoses. It seemed to be an example of fibrosarcoma (Fig. 6).

Although the size and histology of the growth and the necrosis of bone underlying made primary mid-thigh amputation the treatment of choice, his general condition was so bad that it was decided to attempt more by radiotherapy. Lateral and medial fields were used and skin doses of 3300 and 3000 r respectively given using 220 kv. However, only slight response to this therapy could be found on serial biopsy, the tumour cells and stroma remaining as in the first biopsy, but mitoses became less frequent and some collagen was deposited.

His condition deteriorated and he became anuric. His blood urea, reduced by diet and conservative measures, again rose above 200 mg. per cent. Ureteric catheterization and wash-outs produced no urine. Intra-venous isotonic sodium sulphate and bladder irrigation produced no change, and he died of uraemia one month after admission. Post-mortem revealed contracted fibrotic kidneys, an enlarged and flabby heart, pulmonary congestion with areas of bronchopneumonia, but no evidence of sarcomatous metastases could be detected.

Mr. J. R—, aged 76 years, had suffered from bilateral varicose veins with extensive chronic eczema of both legs and occasional ulceration of the left leg for over 30 years. Eighteen months prior to his admission a small lump appeared just above the right internal malleolus and this tumour grew and ulcerated. He presented with a florid fungating growth as shown in Fig. 7. He had never before noticed frank ulceration at this site.

On examination his general condition was excellent. Locally there was a large fungating ulcer over the right internal malleolus with heaped-up rolled edges and a deep

sloughing crater. The whole mass appeared fixed deeply. There was one large, very hard node in the right groin. Extensive varicosities were present in both legs both in the long and short saphenous systems, with much scaling from eczema, and the scars of old ulcers were present on the left leg.

X ray of the right ankle revealed extensive calcification occurring in a soft tissue mass situated over the right internal malleolus, but this calcification did not appear to originate from the underlying bone, which showed only slight reactive periostitis (Fig. 8). Clinically there was no evidence if Paget's disease of bone, and no radiological evidence of this disease could be found in the skull spine or pelvis. The other long bones appeared normal also.

Biopsy was performed and the report read as follows : " Most of this is ulcerated and infected granulation tissue but one corner contains neoplastic tissue. It consists of solid rounded acellular blocks of tissue, staining pale pink with eosin, but a good red with van Gieson, between which columns run small groups of dark-staining polygonal cells, somewhat epithelial in appearance. Except that it is a tumour no certain diagnosis seems possible : the balance of opinion favours osteogenic sarcoma but a strong minority favours carcinoma. Further biopsy would be of assistance " (Fig. 9).

Several further biopsies were taken but no certain diagnosis could be given. Because of the patient's age and at his request cure was attempted by radiotherapy, but it soon became apparent that no regression was occurring and biopsy excision of the node in the groin was considered and performed. This node contained a nodule consisting entirely of masses of woven bone, in the interstices of which were dark-staining polygonal epithelial type cells very similar in appearance to the cells of the first biopsy (Fig. 10).

It was concluded that this tumour represented a most unusual and atypical form of osteogenic sarcoma, and the patient was advised to have a mid-thigh amputation and excision of the inguinal nodes. Because he lived alone and also owned a small personal business he refused to lose his leg above the knee, and we were constrained therefore to perform a below-knee amputation and block dissection of inguinal nodes. This dissection was performed with great thoroughness and a block of areolar tissue and nodes extending up to the bifurcation of the right common iliac artery was removed. Careful sectioning of this mass revealed no other metastasis.

Healing of the amputation flaps by first intention was rapid but the groin wound broke down. He refused skin grafting as he desired to get back to his business and discharged himself. The wound healed by granulation. He remained well for one year, when he was readmitted for treatment of a mass in the posterior flap of the stump. This mass was excised locally, a wide wedge of tissue being removed down to the bone. This local operation was again performed at the patient's insistence. Section of the tumour contained in this block of tissue revealed the identical histological picture presented by the original tumour and the nodule found in the inguinal lymph node. Healing was by first intention. He continued well until March, 1951, when he was admitted to another hospital. He was here discovered to have " cannon-ball " secondaries present in both lungs and he died July, 1951, about 4 years after the first operation, at the age of 82 years.

COMMENT.

There has always been much discussion on the part played by chronic irritation in the aetiology of malignant disease. Whilst there can be no doubt of the significance of such irritants as tar and the carcinogenic hydrocarbons contained in it, of thorium and its bye-products, of radio-active earths, and of many other chemical products or physical energies in the production of malignant change, the position regarding inflammatory irritation is not so clear. Epithelioma of the lower limb is very uncommon in comparison with epithelioma elsewhere. This

rarity must be associated with the fact that the legs are normally covered during adult life and thus protected from actinic radiation. At the same time, it is interesting to note that almost all the recorded cases of lower leg epithelioma have occurred in tissues subjected to years of irritation by chronic inflammatory processes. It would therefore appear that chronic inflammation may be of significance in the causation of cancer, but that the prodromal period is inordinately long.

With special reference to varicose ulcer or eczema it is impossible to disregard the views of Tenopyr and Silverman (1932), who maintain that an incidence of 0·4 per cent of malignant degeneration in such an extremely common condition as chronic varicose veins must indicate that these malignancies occurred by chance and not because of any precancerous propensities in the varicose condition.

We were struck by the observation of Gotheil (1912), which was supported by Knox (1925), that the dermatitis consequent upon varicose veins seems to be more commonly followed by malignant change than frank ulceration. In 2 of our cases the tumours occurred in non-ulcerated skin : that of Mrs. M. W—, in the scar of an old ulcer, and that of Mr. J. R— in deeply pigmented eczematous skin on a leg which had never previously suffered ulceration.

It was not possible accurately to assess the part played by local applications aetiologically—i.e., tar preparations, red lotion and other topical applications designed to increase granulation—because no patient could remember, from the infinite variety of applications tried over the years, what was contained in any of them. No case gave evidence of recent application of known carcinogenic substances.

It seems to us that varicose conditions of the legs, even if present for very many years, should not be considered as in any way pre-malignant. On the other hand progressive growth of the ulcer, the occurrence of haemorrhage, heaping-up or hardening of the edges or a hard node in the groin should be considered as pointers to a malignant change. There should be no hesitation in performing biopsy. There growths do not appear to present the characteristics of epitheliomata elsewhere, being slowly growing, slow to metastasise, and slow to kill, so that early treatment affords an excellent prognosis ; indeed it seems likely that the slow progression of these tumours often remains unremarked and the patient dies with an unrecognised malignant ulcer.

It was our belief, prior to personal experience with this type of case, that the treatment of choice was radiotherapy-controlled by serial biopsy. This form of treatment had been advocated in the literature (Rubenfeld, 1934), and it was in line with the treatment proposed for epitheliomata on other sites. Furthermore the age of the patient made the fitting of an artificial limb and education in the use of such a limb difficult and time-consuming procedures. However our experience with these cases has rather altered our view. In no less than 3 out of the 4 cases amputation had to be advised and, but for the death of one patient, would have been performed. Operation was forced upon us by the fact that, although the malignant disease had been eradicated by radiotherapy, the patients were still made utterly miserable by a large weeping, painful and foul-smelling ulcer showing no signs of healing at all. In 2 cases this condition prevented ambulation and the patient was bedridden. In these circumstances we would, in future, have no hesitation in recommending amputation, below-knee if it is possible to fashion flaps of healthy skin, otherwise mid-thigh, as immediate

treatment. This will prevent loss of time, decrease the period of pain for the patient, and allow the patient to acclimatize himself to the loss of his leg without the disappointment of failed conservative treatment. Radiotherapy should be held in reserve for those patients whose general condition does not permit of operation, and these must be very few now.

The treatment of the inguinal nodes depends upon the age and general condition of the patient. Extensive block dissection is a formidable undertaking in the very old, and in these cases irradiation should be advised. Where the patient will bear the operation this represents the safest method of eradicating growth.

Sarcoma, largely owing to its extreme rarity is difficult to recognise both clinically and histologically. It appears to occur more towards the centre of an ulcer than at the edge, to produce a florid fungating cauliflower growth and to bleed readily. The most common histological type is a fibrosarcoma. These tumours do not react to irradiation and are best treated by amputation. As with the squamous growths, sarcomata in varicose ulcers appear less ready to metastasise than similar tumours elsewhere or in younger patients. This may be due to the fibrosis and scarring present after years of ulceration. The lymph-node metastasis report in the case of Mr. J. R— must be regarded as a pathological curiosity.

In conclusion we consider that this rare condition, if recognised and confirmed by biopsy at an early stage and actively and adequately treated, should have but little deleterious effect on the life expectation of the patient.

SUMMARY.

Six cases of malignant degeneration occurring in varicose ulcer or eczema are reported.

The literature is reviewed, 69 cases of epitheliomata and 3 cases of sarcomata being collected. Four epitheliomata and 2 sarcomata are added. Aetiology and treatment are discussed.

We wish to thank Professor Ian Aird, Miss C. Wood and Mr. M. R. Ewing of the Postgraduate Medical School of London for permission to make use of material pertaining to their patients. We would also wish to thank Professor J. H. Dible and Dr. C. V. Harrison of the Dept. of Pathology for the histological specimens and their reports and Mr. E. V. Willmott, F.R.P.S., for his photographs.

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ADENOCARCINOMA OF THE OESOPHAGUS AND ECTOPIC GASTRIC MUCOSA.

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ADENOCARCINOMA in the oesophagus may arise in three ways: (1) as an upward extension of a carcinoma of the stomach, (2) as malignant change in the mucous glands that are normally found in the oesophageal submucosa, and (3) from ectopic gastric mucosa. The first is not uncommon; the second is rare. The third, also rare, is the type that concerns us here.

In 1950 Carrie reported a case of adenocarcinoma of the oesophagus that had arisen in an area of ectopic gastric mucosa. He reviewed the literature and likened the lesion to the unicorn in that many authors had described it, but only one had ever seen it.

Although he makes no reference to the possibility of a hernia being present, the description of the specimen, coupled with the fact that the tumour was in the cricothyroid region (where ectopic gastric mucous membrane is most frequently encountered—Schridde (1904)), makes it certain that this was indeed a genuine example of this rare lesion.

Bosher and Taylor (1951) describe a case in which ectopic gastric mucosa was present in the oesophagus. There was reflux oesophagitis and stricture formation at the level of the aortic arch. At first sight this would appear to be a case similar to the one about to be described, but it seems more likely that it is another example of hiatus hernia with extreme secondary shortening of the oesophagus, as the barium meal strongly suggests that only a small portion of the stomach is in the abdomen. Although they state that at operation the hernia was small, it is notoriously difficult to distinguish stomach from gullet on external appearances alone. No mention is made of the type of muscle in the resected specimen.

Case Report.

J. R—, male, aged 56.—This patient was admitted to the Middlesex Hospital on 13.i.52 under the care of Dr. Hadley, complaining that food had been sticking behind the lower end of the sternum for 2 weeks. He had no difficulty with fluids. One week prior to admission food stuck in the same place and was regurgitated. Since then solid food would not pass at all.

On examination the patient looked well. There was no wasting or dehydration and no glands were palpable. All systems were normal.

On 14.i.52 an oesophagoscopy was performed (Mr. Douglas Ranger). The oesophagus was found to be obstructed by a hypertrophic ulcerated lesion extending higher up on the anterior wall than the posterior wall. A biopsy was taken

which showed an adenocarcinoma invading the wall of the oesophagus and undermining its squamous lining.

A barium meal on January 28 showed a filling defect in the oesophagus at the level of the bifurcation of the trachea (Fig. 1). This was constant, and caused some obstruction to the flow of barium. The appearances were those of a carcinoma of the oesophagus. No abnormality could be seen in the gastric fundus (Fig. 2). The lower end of the oesophagus appeared normal (Fig. 3). The stomach and duodenum were normal.

The patient was referred to the thoracic surgical department with a view to oesophagectomy.

At operation on 30.i.52 by Mr. T. Holmes Sellors laparotomy was performed. The stomach was found to be completely normal in shape. There was no diaphragmatic hernia present. A small secondary was palpated on the convex surface of the right lobe of the liver. The whole stomach was mobilised and the abdomen was closed. A right thoracotomy was then performed with the patient in a face-down position. A small mass was found just behind the tracheal bifurcation. The oesophagus otherwise appeared completely normal. The mass was dissected from the pericardium posteriorly. The oesophagus was mobilised down to the diaphragm and the stomach was brought up into the chest. The oesophagus was removed from a point 2 inches above the tumour to the cardia and the free end implanted into the upper part of the stomach.

Post-operatively the patient had some fever and pylorospasm but was eventually discharged from hospital well on 4.iii.52.

Pathological report.

Specimen (Fig. 4).—Length of oesophagus 6 inches. One inch from the proximal limit of excision there was a nodular growth encircling the oesophageal wall for a length of 1 inch. It had caused a marked degree of stenosis of the lumen. The mucous membrane above the growth presented a smooth dead white appearance. Below the growth it had a more granular surface, especially just below it and near the distal limit of excision.

Histology.—Section showed an adenocarcinoma of a high grade of malignancy (Fig. 5). The mucous membrane above the tumour was entirely squamous in type and was normal for the oesophagus; that below the growth was entirely glandular apart from a few islands of squamous epithelium about 2 inches above the lower limit of excision. The glandular mucous membrane itself showed variable features; near the distal limit of excision it corresponded closely to a normal cardiac type of gastric mucous membrane, with numbers of parietal cells among the glands. However, most of it showed chronic inflammatory and atrophic changes, with a tendency towards an intestinal type containing many goblet cells (Fig. 6). The appearances were those of a chronic gastritis. The growth itself had arisen at the junction of the squamous and glandular epithelium (Fig. 7).

Heidenhain's stain showed that a few striated muscle-fibres could be found in the wall of the oesophagus between the longitudinal and circular layers of smooth muscle (Fig. 8). These striated fibres could only be found above the level of the growth. In addition it could be seen that the circular muscle coat at the site

of the carcinoma was thickened to form what appeared to be a sphincter. There was no evidence of muscular hypertrophy immediately above the growth.

Spread.—(1) Local: Through all layers of oesophageal wall. (2) Lymphatic: Of 4 glands found, 2 showed involvement by tumour.

DISCUSSION.

Adenocarcinoma is not infrequently encountered in the lower third of the oesophagus, but it is usually found to be of gastric origin with secondary spread up the oesophagus.

After the biopsy report in the case described the possibility of a hiatus hernia was considered, as, if one was present, the carcinoma might well have arisen in an intrathoracic part of the stomach. However, at operation there was never any doubt that the external anatomy of the gullet and stomach were normal, and that there was no hernia.

If, following Barrett (1952), the oesophagus is defined as that part of the alimentary tract lined by squamous epithelium, and the gullet defined as that part which joins the pharynx to the stomach, this patient must be regarded as having a short oesophagus and a normal gullet, for although it is admitted that the oesophageal mucosa may move considerably in relation to its muscle, the squamo-glandular junction in this case was at the level of the tracheal bifurcation.

The presence of striated muscle in the wall of the oesophagus is normal. According to Maximow and Bloom (1937) both muscle layers of the cranial quarter of the oesophagus are composed of striated muscle. In the second quarter the striated muscle is gradually replaced by smooth muscle. The lower half of the oesophagus contains only smooth muscle in its muscular coat. In the case described, striated muscle was present down to and including the site of the adenocarcinoma. This represents a point about 5 inches above the junction of gullet and stomach. The normal gullet is about 10 inches long as measured from the termination of the pharynx at the level of the cricoid cartilage to the cardiac orifice of the stomach. It would thus appear that the striated muscle in this case had a normal distribution. However, the circular muscle-fibres at the squamo-glandular junction appear to have thickened to form a sphincter. This constitutes a departure from normal, and it may have prevented regurgitation of acid secreted by the gastric mucosa lining the lower half of the gullet. This may well have prevented the development of oesophagitis earlier in the patient's life.

There was definite histological evidence in this case that the adenocarcinoma had arisen in the glandular mucous membrane immediately adjacent to the junction of the squamous and glandular epithelium. The emphasis is placed on "glandular", for this showed varied degrees of chronic inflammatory and atrophic changes which obscured the normal picture of a gastric type of mucous membrane. These changes conform to those that may be seen in stomachs that are affected by chronic gastritis, including the atrophic type. Thus we conclude that the lower half of the gullet in this case was lined by gastric mucous membrane that was the seat of chronic inflammatory and atrophic changes, which may well have been a factor in the development of carcinoma.

The rare occurrence of adenocarcinoma in the oesophagus, other than those cases that have obviously spread from a gastric origin, has been put down in the

past to an origin from the mucous glands that are normally found in the oesophageal submucosa at all levels. While not denying that these mucous glands may give rise to carcinoma, it should be remembered that adenocarcinoma of the oesophagus as an entity may arise from islands of ectopic gastric mucous membrane or from grosser congenital abnormalities of the gullet associated with heterotopia.

SUMMARY AND CONCLUSIONS.

(1) A case of a congenital short oesophagus associated with a normal gullet is described. The lower half of the gullet was lined by ectopic gastric mucosa that was affected by inflammatory and atrophic changes. A carcinoma had developed at the squamo-glandular junction. Oesophagectomy was successfully performed.

(2) Adenocarcinoma of the oesophagus is an entity. It may arise from islands of ectopic gastric mucosa or, as in the case described, from a congenital abnormality of the oesophagus associated with a gastric type of mucous membrane lining the gullet.

Our thanks are due to Dr. G. D. Hadley and Mr. T. Holmes Sellors for permission to publish this case, and to Mr. D. Ranger for the oesophagoscopy findings.

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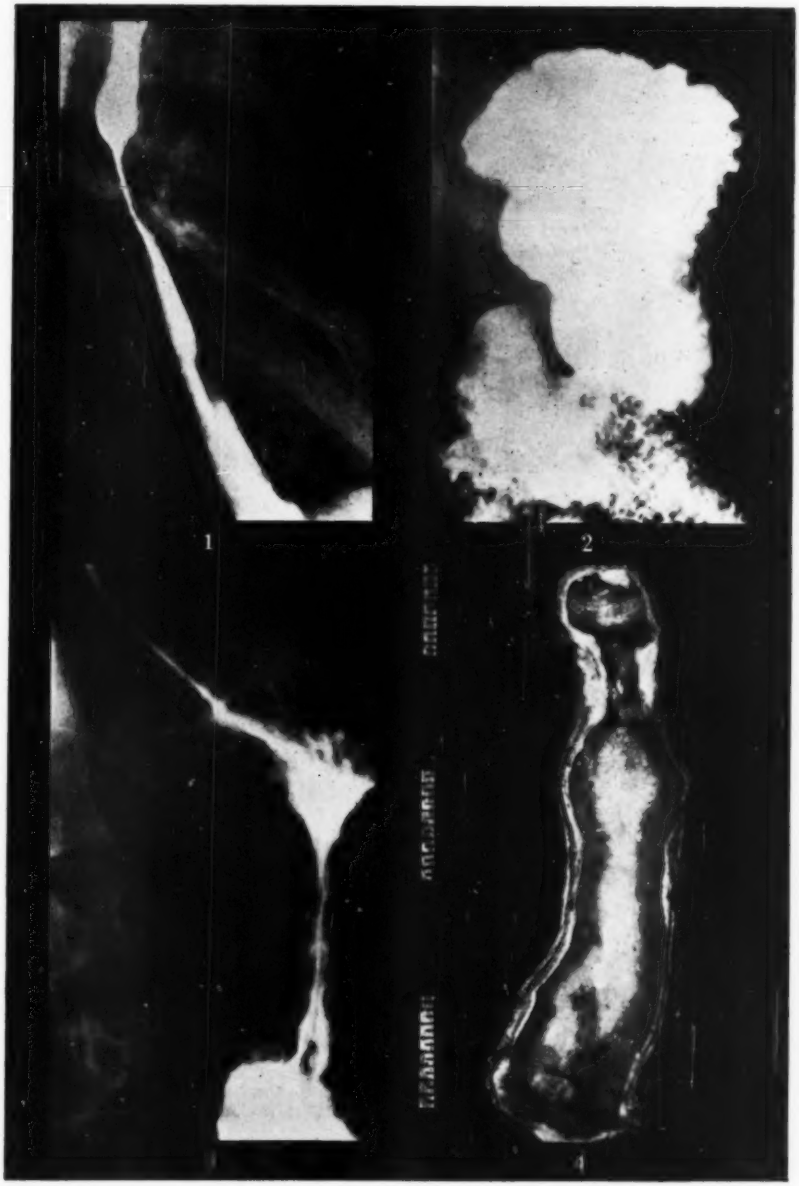
EXPLANATION OF PLATES.

FIG. 1.—Constriction at the level of the tracheal bifurcation, the remainder of the gullet appearing normal.
FIG. 2.—Radiograph taken in the Trendelenberg position with no evidence of a hiatus hernia.
FIG. 3.—Normal mucosal pattern in the gullet.
FIG. 4.—The specimen of resected oesophagus showing a carcinoma at the upper end.
FIG. 5.—Section showing the adenocarcinoma. ×90
FIG. 6.—Section showing the type of mucous membrane seen below the growth. × 90.
FIG. 7.—The squamo-glandular junction showing inflammatory changes with carcinoma infiltrating the submucosa. × 90.
FIG. 8.—Striated muscle-fibre in the wall of the oesophagus. × 450.



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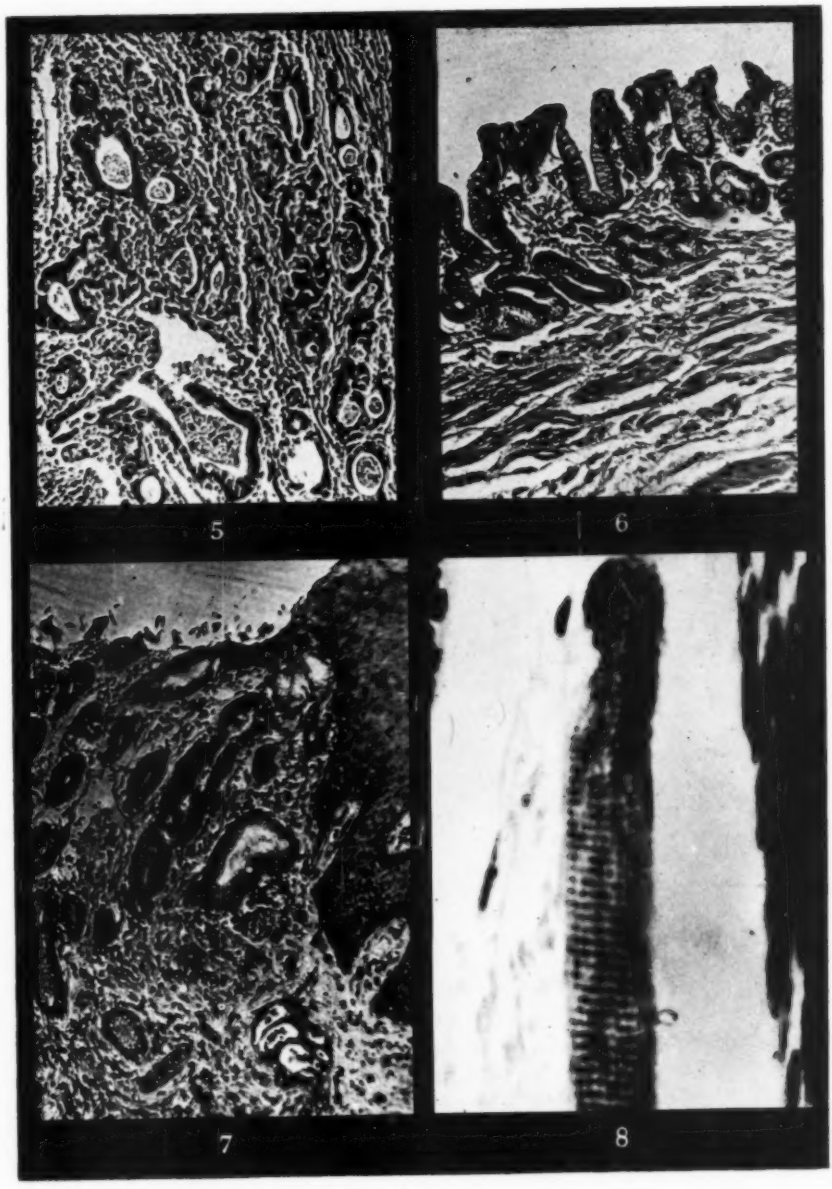

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CANCER AND ESSENTIAL HYPERTENSION.

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In addition to common arteriosclerosis, which, in a certain measure, represents a natural event, we can consider two pathological processes as the main causes of death in people over 40 years of age, namely (1) cancer and (2) essential hypertension. According to statistics at our disposal, not less than about 40 per cent of the population over 40 years of age, and 50 per cent over 50, die of one of these diseases. Both have this in common, that they affect people at the same period of life, i.e., in general beyond the age of 40, bear an absolutely chronic character, and hardly ever show any tendency to spontaneous cure. Moreover, it is typical of both diseases that the earlier in life they make their appearance the more severe the course.

Our investigations arose from the following questions: Are the two diseases interdependent? Are people, who are proceeding along the path of essential hypertension, capable of proceeding simultaneously along that of cancer also? And if this is the case, perhaps only to a lesser extent? If both diseases proceed without excluding each other, and without even being affected by each other, a certain proportion of people suffering from cancer must be found who will display, at the same time, the symptoms of essential hypertension, and the proportion to be expected will have to be in accordance with the hypertension-morbidity of the corresponding age-group.

There are many investigations dealing with the incidence of hypertension, and particularly that of essential hypertension. They are of twofold character: (1) mortality statistics and post-mortem examinations; (2) systematic blood-pressure examinations among people of various ages.

Relying on mortality statistics of the United States, Fahr (1928) estimates essential hypertension to be the cause of death of 23 per cent of the population aged over 50 years, a figure which is held by Volhard (1931) as being too small. Bell and Clawson (1928), on the strength of exact anatomical examinations, came to the conclusion that in all cases of mortality about 7 per cent in the 40 to 49 age-group and 13 per cent of people over 50 years are dying from hypertension. Not included are those cases of hypertension who are dying from any other disease and who are considered by the authors as representing the majority, i.e., 75 per cent of all cases of hypertension; accordingly, hypertension may be met with in 50 per cent of all people over 50 years. Not all of these cases classified as "hypertension" are, however, to be regarded as true "essential hypertension"; a certain proportion very likely belongs to the group of "arteriosclerotic hypertension".

The attempt to come to a definite estimation of the incidence of essential hypertension based on the numerous blood-pressure examinations among various classes of a certain population undoubtedly encounters certain difficulties; one of the main difficulties lies, in my opinion, in the precise definition of essential hypertension. Not every rise in blood pressure, even a permanent one, is, of course, connected with true essential hypertension. It is rather easy to exclude cases of secondary hypertension, such as those caused by Bright's disease, aortic valvular disease and hyperthyroidism; moreover, from a statistical point of view, these cases do not play a major part, at least not in the age-group which is of particular interest to us. Of great importance, however, is the distinction between "essential hypertension" and "arterio-sclerotic hypertension". In the latter the hypertension is, as is generally accepted, due to a lessening of the elasticity of the large vessels, especially that of the aorta, and its incidence in the higher age-groups, i.e., beyond the fifth or even the sixth decade, is a rather frequent one. In contrast to essential hypertension, the arteriosclerotic form is purely systolic hypertension; diastolic pressure in these cases might even be abnormally low. Therefore, it should not be surprising that systematic blood-pressure examinations, taking into account only the systolic pressure, indicate a very high incidence of hypertension, i.e., 50 per cent and more in people over 50 years. Essential hypertension, however, is in the first place a diastolic hypertension, and only cases of hypertension showing either a distinctly high diastolic pressure or a combination of high systolic pressure, and at least moderately increased diastolic pressure, should be regarded as cases of essential hypertension. For instance, while a permanent blood pressure of 150/95 mm. Hg might be due to essential hypertension, a blood pressure of 180/70 mm. Hg might be a sequel of common arteriosclerosis, if diseases such as aortic valvular disease and hyperthyroidism can be excluded.

Taking these facts into consideration the systematic blood-pressure examinations carried out by Master, Marks and Dack (1943) in 15,000 people can be regarded as very helpful in assessing the incidence of essential hypertension. While a systolic blood pressure of 150 mm. Hg and more was found by the authors in a frequency of about 13 per cent in men between the ages 40 to 49, in about 30 per cent in the group 50 to 59, in 50 per cent in the group 60 to 69, and even 60 per cent in the age-group over 70 years (the figures for women are even higher), a considerably lower rate for hypertension was elucidated when based on a high diastolic pressure, i.e., 95 mm. Hg and more. (The diastolic pressure was taken at the complete disappearance of the sound.) The figures found are as follows:

Men.

Age . . .	40–49	50–59	60–69	70–79
Hypertension in percentages .	10·3	20·5	26·6	32·6

Women.

Age . . .	40–49	50–59	60–69	70–79
Hypertension in percentages .	13·3	24·0	29·2	34·0

The people examined by Master, Marks and Dack (1943) were composed of three groups, of whom the two most numerous were (1) industrial workers, and (2) patients of the various departments of Mount Sinai Hospital, New York; though amongst the "ill" people of the hospital the incidence of hypertension was higher than that found amongst the "healthy" workers, the difference was not exceedingly great. Hypertension, i.e., diastolic blood pressure of 95 mm. Hg and more, was found in men between 40 and 49 years among the workers in 8·9 per cent and among the hospital patients in 14 per cent, and between 50 and 59 years among the workers in 17 per cent and among the hospital patients in 21·5 per cent; in women the corresponding figures were as follows: workers 11·3 per cent and hospital patients 15 per cent (40 to 49 years) and 24·0 per cent and 22·8 per cent respectively (50 to 59 years).

Our own statistics are based on 5500 people who, like the hospital cases of Master, Marks and Dack (1943) were suffering from a very large variety of diseases; the figures found are as follows:

Men.

Age . . .	40–49	50–59	60–69	Over 70
Hypertension in percentages .	12·6	22·0	30·0	30·0

Women.

Age . . .	40–49	50–59	60–69	Over 70
Hypertension in percentages .	16·7	31·0	42·0	53·0

In men the figures found by us are very close to those of the American authors; in women, however, they are somewhat higher, at least, as far as the age-group above 50 years is concerned. Two factors may be held responsible for this difference: (1) the persons examined by us were exclusively Jews and, as also accepted by the American authors, the incidence of hypertension among Jews is probably even higher than among other white races; (2) our statistics are based on somewhat different values of blood pressure. While the figures of the American authors are based on the evaluation of the diastolic pressure only, i.e., 95 mm. Hg, ours are derived from a simultaneous evaluation of systolic and diastolic pressure. A person was not classified as suffering from essential hypertension unless, constantly, or at least in most of the examinations carried out, a blood pressure of more than 160/90 mm. Hg was detected, i.e., 165/90 or 160/95 and more. Though a permanent diastolic pressure of 95 mm. Hg may be regarded as a very reliable proof of the existence of essential hypertension, if diseases such as Bright's disease can be excluded, we based our examination nevertheless on a somewhat lower pressure, i.e., 90 mm. Hg; we did so because people suffering from hypertension in the course of their illness may not infrequently display a lowering of their diastolic pressure, probably as a result of developing arteriosclerosis (Fishberg, 1939). The decrease of diastolic pressure from 95 to 90 mm. Hg as a minimum value for essential hypertension will, of course, increase the figure for the morbidity of hypertension; but, apparently, a compensation has been created—at least in men—by relating the diastolic pressure to a minimal systolic

pressure of 165 mm. Hg. (Blood-pressure has always been taken while patient was sitting.) There is no statistical method, based on blood-pressure examinations alone, which will include every case of essential hypertension, or will, with certainty, exclude every other type of hypertension; but this is not of paramount importance, since in view of the high incidence of essential hypertension, small degrees of error will probably not be significant.

It may reasonably be assumed that the figures contained in our statistics, and especially in that of Master, Marks and Dack (1943), indicate fairly accurately the incidence of essential hypertension in the various age-groups; confirmation of this assumption may be noted by the figures contained in the mortality statistics; nor do the anatomical examinations quoted above contradict this.

What, in comparison, is the incidence of essential hypertension in cancer patients? We shall not attach any importance to anything but a great deviation from the normal rate. The number of cancer patients referred to in this paper is 1490; a certain proportion of the cases has been observed by us; as to the remainder, we obtained the necessary data from the cards of the cases put at our disposal by various hospitals of the country. The material comprises all types of carcinoma except that of the skin. Sarcoma is not included for lack of sufficient number of cases. Tables I and II refer to all cases; they indicate the incidence of hypertension found in cancer patients arranged only according to sex and age of the patient.

TABLE I.—*Carcinoma in Men* (579 *Cases*).

Age-group.	Number of cases.	Hypertension found (%).	Usual incidence of hypertension (%).*
40–49	100	3·0	10·3–12·6
50–59	178	5·0	20·5–22·0
60–69	215	14·0	26·6–30·0
70 and more	86	12·8	32·6–30·0

* In all tables the first figure is that of Master, Marks and Dack (1943), the second of the writer.

TABLE II.—*Carcinoma in Women* (911 *Cases*).

Age-group.	Number of cases.	Hypertension found (%).	Usual incidence of hypertension (%).
40–49	289	7·4	13·3–16·7
50–59	307	22·1	24·0–31·0
60–69	251	33·8	29·0–42·0
70 and more	64	50·0	34·0–53·0

As can be seen, there is a great difference between men and women. In women with carcinoma the rate found for essential hypertension does not deviate to any great extent from the normal figure of hypertension morbidity; only in the age-group 40 to 49 is the percentage smaller, i.e., 7·4 compared to the normal figure of 13·3 to 16·7. In men, on the other hand, the deviation from the normal rate is much greater and applies to all age-groups, especially to the two age-groups 40 to 49, 50 to 59; in the group 40 to 49 essential hypertension is met with in 3·0 per cent instead of the normal morbidity of 10·3 to 12·6 per cent, and in the

age-group 50 to 59 it is noted in 5·0 per cent instead of the expected figure of 20·5 to 22·0 per cent.

Do the various types of carcinoma behave in a different way? Tables III, IV and V reproduce the findings in women suffering from carcinoma of the breast, the genitals (ovaries, uterus and vagina), and of carcinoma of the digestive tract (oesophagus, stomach, large intestine and rectum).

TABLE III.—*Carcinoma of Breast in Women* (340 *Cases*).

Age-group.	Number of cases.	Hypertension found (%).	Usual incidence of hypertension (%).
40–49	161	8·7	13·3–16·7
50–59	105	22·8	24·0–31·0
60–69	62	30·6	29·0–42·0
70 and more	12	50·0	34·0–53·0

TABLE IV.—*Carcinoma of Genitals in Women* (222 *Cases*).

Age-group.	Number of cases.	Hypertension found (%).	Usual incidence of hypertension (%).
40–49	66	7·6	13·3–16·7
50–59	87	32·2	24·0–31·0
60–69	53	41·5	29·0–42·0
70 and more	16	50·0	34·0–53·0

TABLE V.—*Carcinoma of Intestinal Tract in Women* (222 *Cases*).

Age-group.	Number of cases.	Hypertension found (%).	Usual incidence of hypertension (%).
40–49	37	2·7	13·3–16·7
50–59	70	17·1	24·0–31·0
60–69	88	30·0	29·0–42·0
70 and more	27	48·0	34·0–53·0

TABLE VI.—*Carcinoma of Intestinal Tract in Men* (379 *Cases*).

Age-group.	Number of cases.	Hypertension found (%).	Usual incidence of hypertension (%).
40–49	64	1·6	10·3–12·6
50–59	111	3·6	20·5–22·0
60–69	145	14·5	26·6–30·0
70 or more	59	10·1	32·6–30·0

In carcinoma of the breast, as well as that of the genitals, the incidence of hypertension almost equals the normal figure; a deviation is to be observed only in the first decade. So far the picture is the same as that reproduced in the general statistics (Table II). In carcinoma of the digestive tract, however, the deviation from the normal figure of hypertension-morbidity seems to be more marked, and not only to be found with regard to the age-group 40 to 49, but to the

50 to 59 group also, though the deviation in the latter group is only of a minor degree (Table V). The number of other types of carcinoma, such as carcinoma of the gall-bladder, pancreas, liver, kidneys and respiratory tract, was not great enough to be used for separate statistical elaboration. For the same reason, in men, only the cases of carcinoma of the digestive tract were statistically elaborated.

Essential hypertension proved to be a rather infrequent occurrence in all age-groups, but especially between the ages of 40 to 59. It is more or less the same picture as that reproduced in the general statistics (Table I).

Though the number of cases of carcinoma of the respiratory tract examined by us was relatively small, we tended to find the same relationship to hypertension as in Table I. We examined 37 cases in the age-group 40 to 59, of whom only one showed hypertension.

Two factors may be held responsible for the rare occurrence of a combination of carcinoma and essential hypertension: (1) carcinoma may have a depressing influence on a previously existing hypertension; (2) a person suffering from essential hypertension may have smaller tendency to develop carcinoma.

The possibility of a depressing action of carcinoma cannot be denied. In this connection we may quote the investigations of Rosenfeld (1929), Feldweg (1929) and Fortunati (1936), who were among the few who carried out systematic blood-pressure investigations in cases of cancer; in the course of the illness a fall in blood pressure could be detected, though the fall, if present, was in most cases only moderate. The examinations took into consideration the systolic pressure only. The number of cases examined by Fortunati (1936) was very small; yet it occurred to him that high blood pressure is more often met with in cases of cancer of breast and female genitals than in cases of cancer of the digestive tract; he failed, however, to take into consideration the normal incidence of hypertension; therefore, he could not even try to answer the decisive question as to whether the frequent occurrence of hypertension in carcinoma of the breast and female genitals or its rare occurrence in carcinoma of the digestive tract are to be considered as extraordinary. A depressant action on blood pressure by carcinoma, as formulated by the authors mentioned above, might be attributed to non-specific factors such as inanition, anaemia, raised temperature and even imposed rest of long duration. It seems unlikely, however, that these factors could be held responsible in any great degree for the rather rare occurrence of carcinoma combined with hypertension in men; after all, in women suffering from carcinoma of the breast and genitals essential hypertension appears in almost its usual frequency, although the non-specific factors mentioned above certainly also constitute part of their disease. Moreover, in order to eliminate these factors as much as possible, we excluded all carcinoma cases with normal or even subnormal blood pressure, if the carcinoma was in an advanced stage or complicated by raised temperature. Especially in men, only those cases were registered whose general condition was good enough to justify at least the trial of a radical surgical treatment; this held true particularly with regard to the cases of carcinoma of the digestive tract. Patients with normal blood pressure but in a less satisfactory general condition were also registered if either the blood pressure existing before the manifestation of cancer was known to be normal or other symptoms such as size of the heart, electrocardiogram and eye fundus were not of such a nature as to arouse the suspicion of a previous hypertension. It must be stressed, however, that both the X-ray examination of the heart and

the electrocardiogram have a restricted value only, since, as it is known, signs of left ventricular hypertrophy may sometimes only be seen after hypertension has been existing for many years. On the other hand, left ventricular hypertrophy does not always allow us to diagnose "essential hypertension," since common arteriosclerosis without hypertension, too, may cause such changes, especially if the people concerned are over 50 or even 60 years of age (Bell, 1946; Fishberg, 1939). Carcinoma cases with hypertension were, of course, always registered, no matter whether their general condition was good or not.

So far only non-specific symptoms accompanying cancer disease such as cachexia, etc., have been taken into account as factors which might exert a negative action on blood pressure. Another question before us is whether cancer itself may act in a depressive sense upon blood pressure by means of specific substances possibly present in its tissue or of catabolics appearing in the cancer patient. If this were the case, it would not be easily understood why hypertension in women with carcinoma of breast or genitals is met with in nearly normal frequency; one could, perhaps, argue that antipressor substances do not necessarily appear in every type of cancer. It is clear, however, that the question as to whether hypertension may be influenced by cancer can unequivocably be answered only by examinations aimed at determining the blood pressure before cancer manifested itself. In our examinations this was possible only in a small proportion of the cases. If only one blood-pressure examination could be secured from the past it was disregarded if it was carried out less than one year or more than three years before the manifestation of the cancer disease. Table VII indicates the percentage of men who suffered from essential hypertension before cancer developed. In view of the small number of cases examined, no differentiation into the various types of cancer seemed advisable; the majority of the cases belonged, however, to the group of cancer of the digestive tract. Moreover, the age-groups were clumped together in two age-groups only.

TABLE VII.

Age-group.	Number of cases.	From hypertension suffered in the past (%).	Usual incidence of hypertension (%).
40–59	72	4·2	15·4–17·3
60 and more	106	21·6	29·6–30·0

As can be seen, men falling ill with carcinoma between the ages 40 to 59 seldom displayed the symptoms of essential hypertension before the onset of the disease; as to the age-groups of over 60 years the result was less conspicuous. This material is certainly not great enough for drawing far-reaching conclusions; it does not allow us to decide whether the rare occurrence of hypertension in certain types of carcinoma and in certain age-groups is attributable alone to a lesser inclination to cancer of people with hypertension, or to a negative mutual correlation which to some extent may also render possible an influence of cancer on hypertension. It is, in my opinion, this research—the investigation of blood-pressure conditions existing before manifestation of cancer disease—which will have to be put on a very broad basis in future.

Essential hypertension and common arteriosclerosis are recognised as quite different processes. In the first years of essential hypertension any anatomical

change typical of arteriosclerosis may be absent and, on the other hand, very severe arteriosclerosis may be found in people with normal or even subnormal blood pressure. Essential hypertension, however, is known as one of the diseases which, like diabetes mellitus, predisposes to the development of arteriosclerosis. If our assumption is correct, that people with normal blood pressure have greater inclination to certain types of cancer, severe arteriosclerosis may be met with less often in cancer patients than in other people. In this connection it may be of interest to learn that Casper as long ago as 1932 noticed that in people with gastric carcinoma the aorta is very often of a character which one would expect to find only in young people ; and while drawing up this text I came across the lately published paper of Wanscher, Clemmeson and Nielsen (1951), who on the strength of systematic anatomical examinations also came to the conclusion that arteriosclerotic lesions are less pronounced in patients suffering from cancer than among non-cancerous persons. As it is known, a much closer correlation exists between hypertension and arteriolosclerosis than between hypertention and arteriosclerosis. Therefore, anatomical examinations purposed to determine the correlation between cancer and arteriolosclerosis might have an even more definite result than those concerning the connection between cancer and arteriosclerosis. According to our results we should expect that the incidence of arteriolosclerosis in cancer patients, and particularly in male patients aged 40 to 59, would be much lower than in other people of the same age.

DISCUSSION.

Simple as the diagnosis of essential hypertension is in evident cases, it may be equally difficult in borderline cases. It might be considered as arbitrary to regard a blood pressure of more than 160/90 mm. Hg as the minimum value for essential hypertension ; but any other limit is hardly less arbitrary. There are authors who consider a pressure as low as 150/90 mm. Hg as minimum value, in which case the number of cancer patients with hypertension would certainly be greater ; but the figure for normal hypertension-morbidity would accordingly increase, and it is hardly to be assumed that a change in the mutual relationship would result.

We feel that our investigations justify the assumption that people suffering from essential hypertension have a lesser inclination to carcinoma ; but we have certainly not put forward any reason for this phenomenon, and any attempt to search for a satisfactory explanation must encounter great difficulties, among which the following may be mentioned : (1) the aetiology of essential hypertension is by no means clear ; it is even not certain whether it has a unitary background ; (2) the fact that in people with hypertension certain types of cancer or cancer of certain age-groups are only seldom met with does not prove that it is the hypertension itself, i.e., its underlying pathological process, which either checks development of cancer or—as can be also inferred from our examinations—delays its manifestation for years or even decades. Possibly an unknown constitutional factor may play a part which is favourable for the development of hypertension but not for cancer or *vice versa*. The possibility, however, that it is the pathological process underlying hypertension which directly checks development of cancer cannot be excluded. Does, perhaps, a competitive action of pressor- and carcinogenic substances come into question ? In view of our

present entirely insufficient knowledge of existence and nature of these substances (steroids ?) we may be permitted to put this question, but we can certainly not expect an answer as yet. Nor is there any answer to the question why the negative correlation between essential hypertension and cancer is more marked in men than in women, especially those suffering from cancer of the breast or genitals. At any rate, at the present stage of our investigations it does not seem practical to search for satisfactory explanations ; after all, the purpose of this report is only to draw attention to certain facts which are in need of further expansion and completion.

SUMMARY.

(1) The incidence of essential hypertension in people suffering from carcinoma is lower than in other people of the same age-group.

(2) The deviation from the usual hypertension rate is more marked in men than in women, and apparently more so in women suffering from carcinoma of the digestive tract than in those with carcinoma of the breast or genitals.

(3) The possible nature of the negative correlation between the two diseases is discussed.

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THE ORIGIN AND THE DEVELOPMENT OF THE " WHITE LABEL " MOUSE STRAIN.

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THE importance of using pure, inbred strains in cancer research is now generally accepted. Strong (1942), emphasizing this point, gave a survey of the origin of some of the best known strains, originating from his laboratory. The new informal document, *Mouse News Letter*, acts as a most valuable source of information as to a great many strains used all over the world.

The characteristics of the different strains as to susceptibility to development of spontaneous tumours, reaction to carcingenic agents, as well as susceptibility to transplantation and other biological responses may be very marked and very different. It is therefore of great importance to have a great number of different strains with marked characteristics, in order to supply research workers with the best material possible for their special needs.

The purpose of the present paper is to describe the origin, development and some of the features of our strain " White Label".

The first animals were obtained from a commercial dealer in Oslo, a young man who used to supply a number of laboratories with albino mice, and who, from time to time, added animals of different origin to his stock. The original mouse was therefore a highly heterozygous animal.

Our strain started with a pregnant female, producing two sons and two daughters, which again formed the first two brother-to-sister mated pairs. Since then brother-to-sister matings have been carried out, only exceptionally the daughter being back-crossed to the father, if a suitable brother was lacking.

Fig. 1 shows the first four generations. A few litters, indicated with small dots, were not carried further. The figure likewise indicates the earliest deviation of the two lines " Line 27 " and " Line 426", which will be described in some detail later. Likewise is indicated what is supposed to be the origin of the branch of the " White Label " strain, which has been kept at the University of Leeds since May, 1934.

In a previous paper (Kreyberg, 1935) some of the features of the tumour reactions of the offspring of one of the two first pairs (2 + 3) have been described. All animals were tar painted after a litter had been raised, and the tar tumour formation as well as the occurrence of spontaneous tumours were recorded. From this procedure a number of data were revealed, and for details the original paper should be consulted.

In the first heterozygous generations the tar tumour reaction was irregular, and a considerable number of mammary carcinomas, lung adenomas and quite a number of soft part sarcomas and a few rarer tumours (osteogenic sarcoma, hypernephroma) occurred.

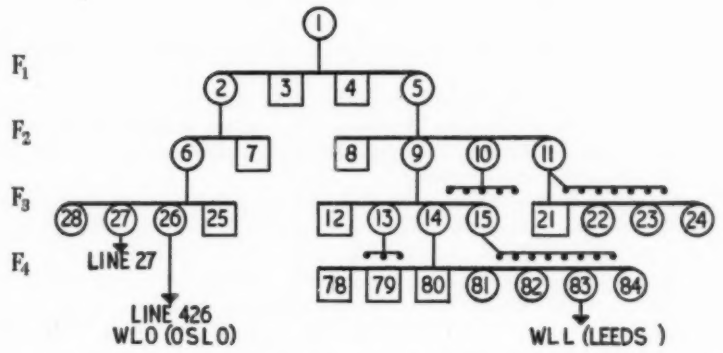

FIG. 1.—The first four generations of "White Label" and the relationship between the Oslo and the Leeds sub-strains.

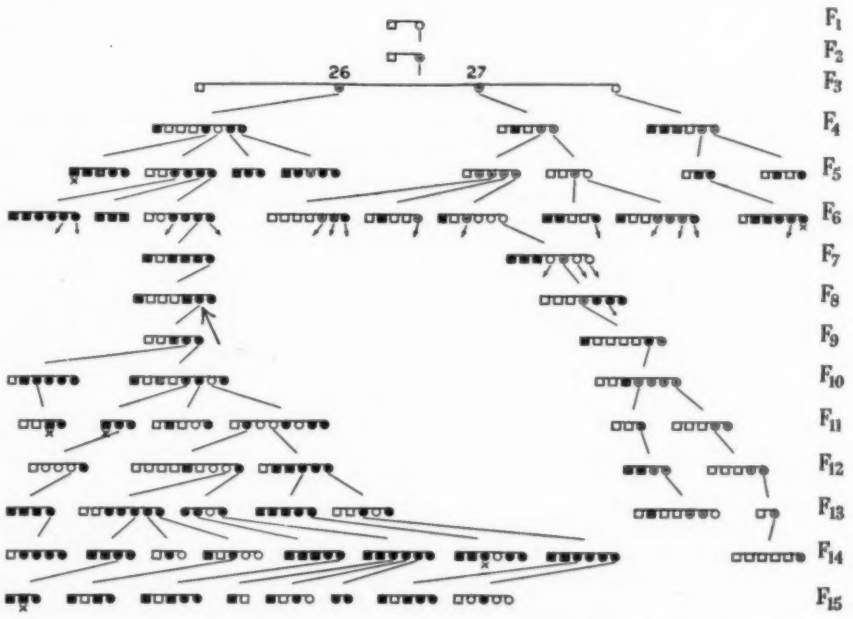

FIG. 2.—Some of the progeny of one of the two first brother-to-sister pairs (No. 2 and 3), and the relationship between the Sub-lines 27 and 426.

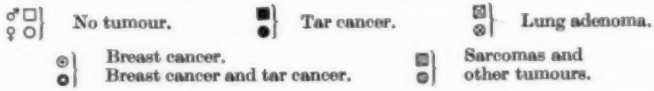

Fig. 2 presents some of the findings, but in the chart not all of the families are included. The figure shows, however, very clearly how two lines were developing with extreme differences as to the formation of spontaneous mammary carcinomas.

" Line 27 " produced a high, and an increasingly higher percentage of mammary tumours. From F_2 and up to and including F_{14}, when this line became extinct, mammary tumours occurred in every generation, and from F_9 until F_{14} 16 out of 18 females developed such tumours.

From the sister of No. 27, namely No. 26, another line was segregated, later designated " Line 426", from the female No. 426, in Fig. 2 indicated by an arrow. In the 11 generations (F_5–F_{15}) in which there were 102 females, not a single instance of mammary carcinoma was observed. Such was the situation in 1935. In the following years the brother-to-sister matings were continued and many sidelines died out, or were not continued, but " Line 426 " was kept until the war interrupted the experiment at F_{37}.

In Table I is given a complete record of the females of all the generations descending from pair (2 + 3) up to F_{37}. In the first column is indicated the generation. In the second column is given the total number of females in each generation. In the third column is given the number of females belonging to " Line 426 "; from F_{15} all the females belong to this line, as this is the only line surviving. In the fourth column is given the total number of mammary carcinomas occurring in each generation. Finally the 24 columns show the number of animals dying in each month after birth, as well as the occurrence of mammary carcinomas, the figures in brackets. At the bottom of the table the sum total is recorded, as well as the reversed figures, indicating the number of females alive at the end of each month.

Table I gives a clear picture of the great number of mammary tumours occurring in the first heterozygous generations. From F_{12} it is only " Line 27 " which still contributes to the presentation of mammary tumours, and from F_{14} when this line becomes extinct, only one single mammary tumour occurs (that is in " Line 426 "). An exception, indicated by a cross, will be mentioned later. This means that " Line 426", during 34 generations, comprising 1595 females, presents one single case of mammary carcinoma. From Table I it is clearly shown that a great many females lived long enough to have been able to develop a mammary tumour according to the usual period of occurrence.

The one mammary tumour, indicated by a cross in Table I, occurred in one female out of 16, belonging to generation F_{19} and F_{20}, injected with folliculine (" Ovifolline", Nyco, Oslo) in weekly doses of 500 mouse units, as previously reported (Kreyberg, 1936).

If we now turn to the other pair of the F_1 (4 + 5) we will find that a few sarcomas occurred in the first generations. These tumours are not marked in either of the tables presented. In Table II, however, the total number of females and their life span, as well as the occurrence of mammary tumours, are recorded in the same manner as for the first pair (2 + 3). It will be seen that in this line a segregation of different sub-lines with marked differences did not take place. In the total population of this line the occurrence of mammary tumours was approximately 21 per cent, and this percentage was kept up to the F_{24} when this line became extinct, due regard being taken of the small number of animals in each generation, and the number of animals dying before the cancer age. If

TABLE I.—*Record of females of all generations decending from pair (2 + 3) up to F37.*

Generation.	♀ All lines.	♀ Line 426.	Mammary carcinomas.	Animals Dying and Breast Tumours Occurring for Each Month after Birth																							
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
F_1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–
F_2	1	–	1	–	–	–	–	–	–	–	–	–	–	(1) 1	–	–	–	–	–	–	–	–	–	–	–	–	–
F_3	3	–	2	–	–	–	–	–	–	–	–	–	–	(1) 1	–	–	–	1	–	–	–	–	–	–	–	–	(1) 1
F_4	8	–	5	–	–	–	–	1	–	(1) 1	–	–	–	–	(1) 1	–	–	(1) 2	(1) 1	1	–	(1) 1	–	–	–	–	–
F_5	20	4	6	–	–	–	–	–	–	–	–	–	(1) 2	(1) 3	1	(1) 1	1	1	(1) 1	(2) 7	–	1	2	–	–	–	–
F_6	25	5	8	–	–	–	–	–	–	–	(1) 1	–	–	(1) 4	(1) 2	4	(2) 2	1	(1) 2	(1) 1	1	1	(1) 1	1	1	1	1
F_7	37	1	9	–	–	–	1	2	–	–	–	–	–	1	(2) 8	(1) 3	2	(2) 3	5	1	1	3	–	(4) 4	2	1	2
F_8	56	2	10	–	–	2	–	1	2	1	1	–	2	1	1	(1) 3	4	(3) 8	(3) 5	(1) 4	(1) 4	2	7	5	(1) 3	–	–
F_9	53	4	16	–	–	–	3	1	4	–	(1) 1	(1) 1	–	–	(2) 3	1	(1) 3	(1) 3	(3) 9	(3) 3	(1) 2	(2) 6	5	4	(1) 3	1	–
F_{10}	30	8	7	–	–	–	1	–	–	–	–	(1) 1	–	1	(2) 6	(1) 2	–	5	2	(1) 4	(2) 5	–	–	4	–	–	1
F_{11}	22	12	5	–	–	1	–	1	–	–	–	–	1	–	(1) 2	(2) 3	(2) 5	2	–	–	2	–	2	1	–	–	1
F_{12}	17	10	4	–	–	1	1	1	–	–	–	–	–	–	(1) 1	2	(2) 2	(1) 2	2	3	–	1	2	1	–	–	1
F_{13}	19	15	3	–	–	–	–	–	–	–	–	1	(2) 2	(1) 1	1	3	2	3	1	1	–	–	2	2	–	–	–
F_{14}	26	25	1	–	1	–	2	–	–	–	–	1	1	1	–	(1) 4	5	2	1	2	1	2	1	2	–	–	–
F_{15}	14	14	–	–	–	–	4	–	–	–	–	–	–	–	1	3	3	2	–	–	–	1	–	–	–	–	–
F_{16}	17	17	–	–	–	1	–	1	–	–	–	–	–	1	2	7	3	–	–	–	–	–	1	1	–	–	–
F_{17}	18	18	–	–	–	–	2	–	–	–	–	1	2	1	–	5	2	3	1	–	1	–	–	–	–	–	–
F_{18}	22	22	–	–	1	1	3	–	1	–	1	–	2	–	1	1	4	2	3	–	1	–	1	–	–	–	–
F_{19}	29	29	–	–	–	–	2	1	–	–	1	–	1	–	3	3	2	4	3	1	5	1	1	1	–	–	–
F_{20}	31	31	×	–	–	1	1	–	–	1	1	2	–	3	–	×7	7	3	–	3	1	–	–	–	–	–	1
F_{21}	30	30	–	–	–	1	–	1	–	–	1	–	2	–	1	4	6	2	3	1	2	1	2	3	–	–	–
F_{22}	36	36	1	–	–	3	2	–	–	–	–	–	1	(1) 2	4	3	6	3	3	2	2	2	1	2	–	–	–
F_{23}	40	40	–	–	–	2	1	–	1	–	1	1	–	–	3	4	1	5	4	6	–	1	3	2	–	–	–
F_{24}	56	56	–	–	–	2	3	6	–	2	1	–	1	–	–	5	5	–	14	8	1	2	1	1	2	2	1
F_{25}	70	70	–	–	–	7	7	1	–	–	–	1	2	2	4	6	5	14	4	3	6	5	2	1	–	–	2
F_{26}	90	90	–	–	4	4	2	6	1	3	–	–	–	4	8	10	18	11	8	3	3	4	1	–	–	–	–
F_{27}	92	92	–	–	–	2	1	4	2	3	3	1	2	4	12	14	17	12	10	2	3	–	–	–	–	–	–
F_{28}	135	135	–	–	4	2	7	7	5	2	1	3	2	8	11	19	26	16	8	7	5	2	–	–	–	–	–
F_{29}	123	123	–	–	3	5	3	5	4	5	–	1	1	4	2	19	26	16	10	9	2	3	4	1	–	–	–
F_{30}	115	115	–	–	1	4	3	6	–	1	3	5	1	1	8	9	21	22	16	9	3	1	1	–	–	–	–
F_{31}	111	111	–	–	2	4	3	1	2	1	5	1	2	4	10	18	17	27	5	4	1	4	–	–	–	–	–
F_{32}	91	91	–	–	–	2	5	1	5	5	1	2	1	2	4	7	20	17	9	3	4	1	2	–	–	–	–
F_{33}	98	98	–	2	9	5	2	8	1	2	–	–	–	–	5	9	18	20	12	3	–	1	1	–	–	–	–
F_{34}	77	77	–	–	7	7	6	6	2	–	1	–	–	–	5	12	9	12	6	3	1	–	–	–	–	–	–
F_{35}	77	77	–	–	2	11	15	11	4	–	–	–	–	1	1	5	10	14	3	–	–	–	–	–	–	–	–
F_{36}	88	88	–	–	16	18	25	12	1	1	–	–	2	–	–	–	6	5	1	1	–	–	–	–	–	–	–
F_{37}	49	49	–	–	17	16	3	4	2	1	1	1	1	–	–	–	3	–	–	–	–	–	–	–	–	–	–
Total	–	1595	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	1827	–	–	2	67	102	108	88	37	29	24	23	31	51	111	196	262	243	152	95	57	47	43	32	11	7	9
	–	–	(78)	–	–	–	–	–	–	(1)	(2)	(2)	(3)	(6)	(10)	(7)	(7)	(8)	(9)	(8)	(4)	(3)	(1)	(4)	(2)	–	(1)
Animals alive				1825	1758	1656	1548	1460	1423	1394	1370	1347	1316	126	1154	958	696	453	301	206	149	102	59	27	16	9	0

TABLE II.—*Record of females of all generations descending from pair(4 + 5) up to F24.*

Generation	♀ All lines	Mammary carcinomas	Animals Dying and Breast Tumours Occurring for Each Month After Birth																						
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
F_1	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F_2	3	–	–	–	1	–	–	1	–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–
F_3	14	5	–	–	–	4	–	–	–	–	–	–	1	2	(1) 2	–	(1) 2	–	–	(1) 1	(1) 1	(1) 1	–	–	–	–
F_4	14	4	–	–	–	–	–	–	–	–	–	–	1	2	–	(1) 3	(1) 2	(1) 2	(1) 2	–	2	–	–	–	–	–
F_5	15	4	–	–	–	–	–	–	–	–	–	1	–	1	(1) 3	2	1	1	(1) 2	(1) 2	–	–	–	1	1	–
F_6	16	6	–	–	–	–	–	–	–	–	(1)	1	2	2	2	(1) 1	–	–	(2) 2	2	(1) 2	–	(1) 1	–	–	1
F_7	14	7	–	–	–	–	–	–	–	–	–	–	1	1	(2) 4	(1) 2	1	–	(2) 2	(1) 1	(1) 1	–	–	1	–	–
F_8	29	12	–	–	–	–	–	1	–	–	2	–	1	2	5	4	3	4	(1) 4	–	–	(1) 1	2	–	–	–
F_9	25	5	–	–	–	2	2	–	–	(1) 1	–	2	–	(1) 2	1	2	(2) 3	3	1	2	2	–	–	(1) 1	–	1
F_{10}	21	6	–	–	–	–	1	–	–	–	–	–	1	4	3	(1) 4	(1) 1	–	(1) 2	(2) 4	–	–	–	–	(1) 1	–
F_{11}	27	4	–	–	–	3	5	1	–	–	–	–	–	2	2	1	(2) 2	–	(2) 4	1	3	2	1	–	–	–
F_{12}	16	3	–	1	1	–	–	–	–	–	1	–	1	1	2	3	(1) 1	1	(1) 2	(1) 1	1	–	–	–	–	–
F_{13}	8	1	–	–	–	1	–	–	–	–	–	–	–	1	1	–	–	2	2	–	(1) 1	–	–	–	–	–
F_{14}	11	–	–	–	–	–	–	–	–	1	–	–	1	2	2	2	1	2	–	–	–	–	–	–	–	–
F_{15}	5	–	–	–	1	1	–	–	–	–	–	–	–	–	2	–	–	–	–	1	–	–	–	–	–	–
F_{16}	6	1	–	–	–	–	–	–	–	–	–	–	–	1	3	(1) 1	–	–	–	–	–	1	–	–	–	–
F_{17}	10	2	–	–	1	–	–	–	–	–	–	1	1	1	(1) 5	–	(1) 1	–	–	–	–	–	–	–	–	–
F_{18}	12	2	–	–	–	–	–	–	–	–	–	–	(1) 3	(1) 2	5	1	–	–	–	1	–	–	–	–	–	–
F_{19}	4	3	–	–	–	–	–	–	–	–	–	(1) 1	–	–	(1) 1	1	–	–	(1) 1	–	–	–	–	–	–	–
F_{20}	9	3	–	–	–	–	–	–	–	–	–	2	1	(1) 1	–	(2) 3	1	–	1	–	–	–	–	–	–	–
F_{21}	13	1	–	–	–	1	–	1	–	–	1	–	–	1	3	3	–	(1) 1	1	1	–	–	–	–	–	–
F_{22}	8	–	–	–	–	–	–	1	–	–	–	–	1	3	1	–	1	–	1	–	–	–	–	–	–	–
F_{23}	1	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	(1) 1	–	–	–	–	–	–	–	–	–
F_{24}	3	–	–	–	–	–	2	1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Total	285	–	–	1	4	13	10	6	–	2	4	8	15	32	47	33	21	16	27	17	13	5	4	3	2	2
		(60)	–	–	–	–	–	–	–	(1)	–	(2)	(1)	(3)	(6)	(7)	(10)	(2)	(12)	(7)	(4)	(2)	(1)	(1)	(1)	–
Animals alive			285	284	280	267	257	251	251	249	245	237	222	190	143	110	89	73	46	29	16	11	7	4	2	0

only females reaching 12 months of age or more are considered, the mammary carcinoma frequency was approximately 35 per cent.

From several publications it will be known that a strain, designated as " White Label," has been used in Leeds for many years, and from Leeds sent to other laboratories. The data available clearly show a very great difference between the Oslo and the Leeds strains of " White Label ". As confusion may be caused, I find it desirable to try and analyse these differences, and possibly explain their background.

Dr. G. Bonser, University of Leeds, wrote me in August, 1949, regarding this question as follows :

" I still keep ' White Label ' mice in this laboratory. By suckling offspring of low-cancer mothers of another strain on ' White Label ' females I showed that the milk factor was present. By suckling ' White Label ' babies on RIII mothers I showed that they were susceptible to the RIII milk factor, which I regard as a potent factor. I never published these experiments, and they were only done on small numbers of mice, but they point strongly to the fact that ' White Label ' mice in Leeds have the milk factor, and are susceptible to it."

Dr. Bonser wrote me a further letter in February, 1951, stating that—

" Mammary cancers still occur in our line and we regard the incidence as being about 30 per cent in mice which live to be 12 months of age or more, but I have not any recent accurate estimate of the mammary cancer incidence, because we have been very short of accommodation lately. . . . "

Addition :

" Now, in 1952, we find that 7 of 33 females over 10 months of age have developed mammary cancer, usually rather late in life. But we make no attempt to assess the mammary cancer incidence accurately as we usually dispose of breeding females when they reach the age of one year."

I also had the following information, dated January, 1951, from Dr. W. S. Bullough :

" My experience with these mice falls into two periods. First when I was working in the Zoology Department in Leeds and when I obtained the mice from Dr. Bonser. Then mammary carcinoma was common in females of from 11 months of age onwards. I have no figures now, but my recollection is that it developed in not less than 40 per cent of the older females. Also there was the odd occurrence in a male, though this was of course rare."

Further :

" This colony was allowed to die out in 1944, but in 1946 I obtained two females and a male from Dr. Bonser and started a new colony in the University of Sheffield. Since then a colony of some 200–300 mice has been maintained. . . . Thus I have had plenty of opportunity to observe the incidence of mammary carcinoma in older females, and I have found it to be common—much commoner it seems than in Dr. Bonser's parent strain in Leeds."

Further, in 1952 :

" The final chapter in the story starts in 1949 when, in the autumn, I took the milk factor out by fostering one family with a Strong's CBA female. The family was born in an open grid and the babies fell through into a lower compartment. My present colony is entirely derived from this one family, and now mammary cancer is exceedingly rare. Indeed I cannot remember when I last had a case. So my present stock of your mice is without the milk factor, and I suppose it is no use for your own present purposes."

I regret very much that I cannot definitely state so, but I have strong reasons to believe that the animals sent to Leeds in May, 1934, were descendants from the pair (4 + 5) of my " White Label ". This would offer a very natural explanation of the great similarity between the Leeds strain and one of the main branches of my strain under development in 1934. The animals sent to Leeds probably belonged to the F_{16} of this branch, described above, and the mammary tumour characteristics are very similar. If this supposition is correct, it would establish a very formal and remote relationship only between the Oslo and the Leeds strains of " White Label ". Actually, they would have only the completely heterozygous founding mother in common.

Whereas the branch (4 + 5) has shown a remarkable stability from the very beginning until it became extinct in Oslo but was still maintained in England, the branch (2 + 3) at an early date started to segregate into very different lines with gradual stabilisation of their characteristics. The present only surviving line (426) in Oslo, through one influence or another, lost the milk factor probably at an early date. In a series of experiments a few years ago the milk factor was reintroduced into the Oslo " White Label " strain, through suckling a breast cancer female of another origin.

SUMMARY.

A brief account is presented of the origin and the development of the " White Label " strain. Starting with a pregnant heterozygous female and through subsequent systematic brother-to-sister matings a non-selective segregation took place, resulting in the formation of two main branches and several lines. A description of some of these lines is given. At present one " White Label " strain (Leeds) carries the milk factor and produces about 30 per cent of mammary carcinomas in females reaching the age of 12 months or more. This strain has the characteristics of the branch (4 + 5) of the original material. Another " White Label " strain(Oslo) is devoid of the milk factor and produces practically no mammary carcinomas spontaneously. This strain is probably one of the lowest mammary cancer strains in existence. For future references it is therefore of great importance to distinguish between these two " White Label " strains ; therefore the Leeds strain will be designated WLL and the Oslo strain WLO. The two strains most probably have only a heterozygous founding mother in common.

This experiment has been carried out for more than twenty years. It would not have been possible without the assistance of my collaborator Miss Hj. Aasland, who continued the experiment during the first years of the war, until illness and an untimely death ended her devoted and loyal partnership. During the remaining years of the war the strain was maintained at a reduced scale through the initiative and faithful service of Mrs. E. Wraal.

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THE ADRENAL GLANDS IN SPONTANEOUS LYMPHOMATOSIS IN BIRDS.

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SEVERAL authors have shown that the adrenal glands play, or may play a part in the changes associated with tumour growth.

Sokoloff (Sokoloff, 1931 ; Sokoloff and Arons, 1940) observed that the adrenal glands increased up to three times their normal size or even more in the case of Rous sarcoma in birds. There was a positive correlation between adrenal size and tumour size. The enlargement concerned chiefly the cortex. Histological examination indicated two different stages : a primary stage with hyperactivity, and a secondary one with hypoactivity, lipoid storage and fatty degeneration.

Enlarged adrenal glands were further observed by Oike (1930) in rabbits with tar cancer and by Tamura (1934) in rats with Flexner-Jobling's carcinoma and in rabbits with Kato's sarcoma. It is evident from Tamura's tables that the medulla (in the cancer rats) underwent no enlargement, but actually decreased a little in volume. The average increase of weight of the whole gland amounted to about 40 per cent of normal weight in rats with the relatively benign carcinoma and to about 68 per cent in rabbits with the very malignant sarcoma.

Okamoto (1935) working with Kato's sarcoma in rabbits also found a marked increase of the whole adrenal gland which was entirely due to cortical enlargement, whereas the medulla after an initial stage of some swelling decreased in volume. Substances in the cortex staining red by Sudan III diminished in later stages of tumour growth. The changes of the glands due to sarcoma growth could largely be reproduced by repeated injections of alcoholic extracts of the sarcoma and of human uterine cancer. Also here the medulla showed atrophy after initial swelling. An interesting observation was that the injections gave the described adrenal picture also in hypophysectomised animals, whereas non-injected animals showed marked adrenal atrophy after removal of the hypophysis.

Ball and Samuels (1938), Sure, Theis and Harrelson (1939), and Haddow and Horning (1950) working with Walker carcinoma observed adrenal enlargement (Ball and Samuels, and Sure, Theis and Harrelson: about 90 per cent increase). Contrary to Okamoto (1935), Ball and Samuels (1938) did not find corresponding enlargement after hypophysectomy.

In lymphocytic leukaemia in rats, Donald and Higgins (1951) observed marked hypertrophy of the adrenal cortex.

We have traced no accounts of the general behaviour of the adrenal glands in lymphomatosis in birds.

Elliot and Tuckett (1906) determined the ratio of cortex : medulla (areas) in

normal birds to be about 1:1. Their figures do not indicate any difference in this ratio as between the sexes as is the case in man. Contrary to the condition in mammalians, the adrenal glands of the fowl do not increase with growth after adolescence.

EXPERIMENTAL.

Material.

Adult birds, mainly hens, were examined. The number of cockerels was too small to admit a proper comparison between sexes. In all examinations concerning weight, ratio of cortex : medulla and cholesterol content, however, the examined cockerel adrenals did not differ from the female ones. Each group in the following, control as well as lymphomatosis groups, thus included one to three cockerels.

Elliot and Tuckett (1906) found no positive correlation between adrenal weight and body weight in adult birds, and our figures support their observations. If the normal birds are equally divided according to body weight into one light and one heavy group, the former had on the contrary some higher mean adrenal weight. The gland weights are therefore given in absolute figures.

About half of each examined group was composed of White Leghorn birds, while the other half consisted of Rhode Island Reds, Barred Plymouth Rocks and crossbreds. As was stated concerning sexes, this material did not indicate differences between breeds. Still, if such differences exist, which may very well be demonstrated with bigger breed groups, their effect ought to have been fairly eliminated in this investigation by similar breed distribution within the compared groups of birds.

Control birds as well as lymphomatosis birds were collected during the same seasons—autumn and winter. The controls were chosen as apparently healthy animals and their freedom from disease was confirmed at the following post-mortem examination. The lymphomatosis birds were selected from material sent in from breeders for post-mortem examination. Only cases with marked features of visceral or neural lymphomatosis were selected. Special care was taken that *both* of the adrenal glands were free from lymphoid infiltration. Single microscopical clusters of lymphoid cells, however, did not exclude the case from examination.

All lymphomatosis birds and most of the control birds were in a non-laying state.

METHODS.

Determination of the cortex : medulla ratio.

The left glands were removed from half of the birds in each of the control and lymphomatosis groups. The right glands were removed from the remaining birds of these two groups. They were then fixed in 10 per cent formalin in physiological saline, then frozen in CO_2 and cut to 15μ thick sections. After staining in Ehrlich's haematoxylin and Sudan IV the section was photographed at low magnification (about 30 times). This admitted roughly one-third or more of the cross-section area to be exposed. In all cases part of the gland periphery was brought into the picture. As is well known, the cortex in the fowl is arranged as a great number of islands and strands. The peripheral islands are often

bigger than the more central ones, with an arrangement that recalls the zona glomerulosa in mammals. In order to reduce the error produced by the uneven distribution of the cortex within the gland, part of the gland periphery was thus always made part of the picture.

The picture was then enlarged to an area of about 300 cm.^{2} The total area, with the fibrous capsule and notable vessels excluded, and the area of the cortex were determined by planimetry. The medullary area was computed as total area minus cortical area, and the ratio of cortical area : medullary area was determined.

From each gland, i.e., from each case, three sections were prepared as described. One was taken from the middle part of the gland, and two others from near the two gland poles, respectively.

Cholesterol determination.

Glands were taken as for the planimetrical determination, i.e., in half of the cases the left and in the other half the right gland. The method described by Bannerjee and Deb (1951) was used with the modification that the glands were extracted with 50 c.c. of acetone (Soxhlet apparatus) instead of 8 c.c. and that one-tenth of the extract was taken to colour development.

Statistical treatment.

Analysis of variance and the comparison of two means were used according to well-known methods. P (probability of random action only) $<0{\cdot}001$ is marked *** and regarded as highly significant. $P>0{\cdot}05$ is regarded as not significant.

RESULTS.

Adrenal gland weights. (Table I.)

TABLE I.—*Weight of Both Adrenal Glands (mg.).*

	Controls.	Lymphomatosis.	Visceral lymphomatosis.	Neural lymphomatosis.
Number of birds	21	39	27	12
Mean weight of both adrenal glands (mg.)	167·429± 8·769	230·667± 7·180	237·259± 8·939	216·833± 11·273
Mean body weight (kg.)	2·21	1·84	1·95	1·61

The difference between gland weights of lymphomatosis birds and normal birds, $63{\cdot}238 \pm 11{\cdot}334$, is highly significant ($t = 5{\cdot}579$*** for $df = 58$). The corresponding difference between visceral and neural lymphomatosis is not significant.

Ratio cortex : medulla. (Table II.)

The results in Table II are based on altogether 60 planimetrical computations, namely, three for each of the 20 birds. The ratio cortical : medullary volumes was calculated in the following way : if the ratio between the areas is $a : b$, the ratio between corresponding volumes is $\sqrt{a^3} : \sqrt{b^3}$.

TABLE II.—*Cortex : medulla Ratio and Cortical Volume in Percentage of Total Gland*

	Controls.	Lymphomatosis.
Number of birds	10	10
Ratio cortical : medullary areas	0·971 : 1	1·537 : 1
Ratio cortical : medullary volumes	0·957 : 1	1·907 : 1
Cortical volume in percentage of total gland volume	48·901	65·600

Statistical treatment.

A statistical treatment (analysis of variance) of the planimetrical results is shown in Table III.

TABLE III.—*Analysis of Variance of Cortex : Medulla Ratios.*

Cause of variation.	*df.*	Sum of square.	Mean square.
Between diagnostic groups	1	4·811	4·811 S^2B
Within diagnostic groups	58	5·012	0·086 S^2w
Between birds	18	3·878	0·215 S^2b
Within birds	40	1·134	0·028 ("error") S^2w
Whole sample	59	9·823	0·209

Comparison of mean square : $\frac{S^2b}{S^2w} = 7{\cdot}679^{***}$.

$$\frac{S^2B}{S^2w} = 171{\cdot}821^{***}. \quad \frac{S^2B}{S^2b} = 22{\cdot}377^{***}.$$

The quotient $\frac{S^2b}{S^2w}$ shows that the difference between birds is highly significant. The difference between diagnostic groups (controls and lymphomatosis birds) is, according to the quotient $\frac{S^2B}{S^2w}$, also highly significant, and according to the quotient $\frac{S^2B}{S^2b}$ of considerably greater importance than the variation between birds.

Cholesterol determinations.

The results, based upon 12 normal and 12 lymphomatosis birds, are shown in Fig. 1.

The mean cholesterol content per gland was $4{\cdot}450 \pm 0{\cdot}469$ mg. in the control (mean gland weight 90·833 mg.) and $7{\cdot}525 \pm 1{\cdot}062$ mg. in the lymphomatosis birds (mean gland weight 121·667 mg.). The distribution of absolute cholesterol values is shown in A.

In B the cholesterol values are made corresponding to 100 mg. gland weight.

Now the lymphomatosis group lies only somewhat higher than the control group (mean is 6·185 mg. as against 4·899 mg.).

In c the cortical weights have been calculated by using the volume per cent figures from Table II, which should be approximately correct. The cholesterol values are plotted against 100 mg. cortex weight. Here the mean of the lymphomatosis group is slightly lower than the mean of the control group (the mean is 9·428 mg. as against 10·018 mg.), and most of the dotted curve lies below the solid one.

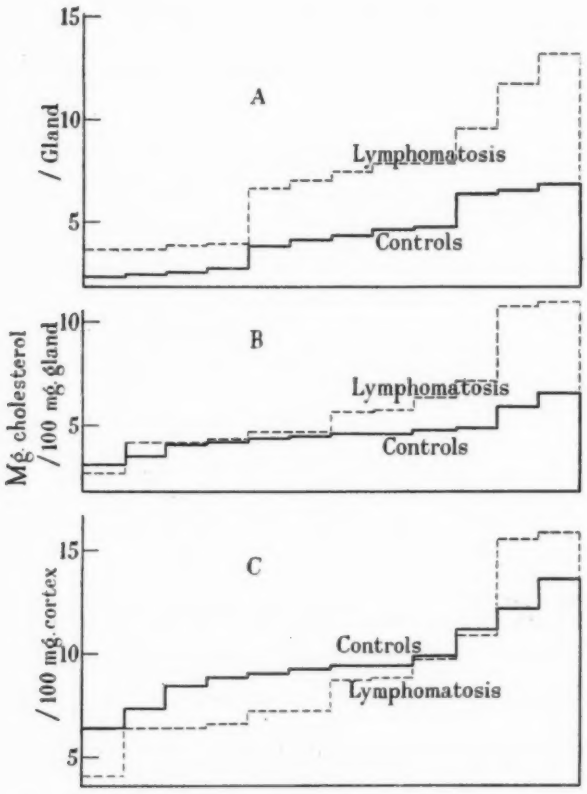

FIG. 1.—Results of cholesterol determinations in controls and lymphomatosis birds.

CONCLUSIONS AND DISCUSSION.

It is evident that lymphomatosis in birds runs with enlargement of the adrenal glands (Table I) which is not caused by tumour infiltration. The enlargement in this material reached an average of nearly 38 per cent of normal gland weight, i.e., much less than that which Sokoloff (1931) found in Rous sarcoma and even less than the enlargement observed in some mammalian tumours. The enlarge-

ment was more marked in the visceral form than in the neural form of the disease. The difference between the two tumour forms, however, was not significant in this material, which may be due to the use of too few birds. It does, however, suggest, as Sokoloff (1931) found in the Rous sarcoma, the existence of a positive correlation between adrenal enlargement and tumour size. The tumour size in neural lymphomatosis is always considerably less than in the visceral variety.

The measurements of cortical and medullary areas clearly show that the enlargement is entirely caused by increase of the cortex. From Tables I and II it can be calculated that the average cortical weight in lymphomatosis is about 150 mg. for both glands as against 82 mg. in the normal birds, i.e., the cortex has increased by 83 per cent. The medullary weight is calculated to 81 mg. in lymphomatosis and to 85 mg. in the controls. Thus the medulla does not increase but may slightly decrease in weight. This is in accordance with the observations of Okamoto (1935) and others in the matter of mammalian tumours which have passed the first stage.

The cholesterol determinations show that the volume increase of the adrenal glands, i.e., of their cortex, is paralleled by an absolute increase of cholesterol, the supposed precursor of steroid production (Fig. 1A). When the cholesterol content is related to the gland weight, however, the increase is less marked (Fig. 1B), and when related to a unit of cortex the cholesterol in lymphomatosis is in fact decreased. This is more evident from Fig. 1C than from comparison of the means, as two lymphocytosis cases showed exceptionally high cholesterol values and thus brought the mean to a higher level than that characteristic of the majority of cases. The impression was that lymphomatosis in a more or less advanced stage is characterised by a big adrenal cortex which in most cases is relatively poorer in cholesterol than normal cortex. The histological examination now and then showed cases of lymphomatosis with considerable disappearance of sudanophil substances but hardly revealed the slight but systematic deprivation as did the chemical determination.

As will be shown in a later paper, acutely running experimental lymphoid tumours in chicks (a transplantable strain of lymphoid tumour derived from a case of spontaneous lymphomatosis) runs with a still more marked depletion of cholesterol than spontaneous, chronic lymphomatosis as well as with adrenal enlargement. This would correspond with Sokoloff's first stage of adrenal hyperactivity in Rous sarcoma. He concluded that the second stage was one of hypoactivity and lipoid storage.

The results of our investigation indicate, however, that in avian lymphomatosis the second stage is also characterised by hyperactivity. A less probable alternative explanation would be that the cortex does not grow as a consequence of increased demand of steroids but because of difficulties in producing them. On the contrary, the parallel with Selye's (1950) general adaptation syndrome with increased adrenal activity first during the alarm reaction and later in the stage of resistance is obvious. The adrenal changes in lymphomatosis should then be considered as an answer to non-specific stress.

SUMMARY.

The adrenal glands in lymphomatosis birds were examined by weighing and determining the proportion of cortex and medulla and the cholesterol content.

1. In lymphomatosis the adrenal glands increase their normal weight by about 38 per cent.

2. The enlargement is caused by increase of the cortex. The medulla may on the contrary slightly decrease.

3. The cholesterol content per gland increases, but when measured in terms of concentration per unit of cortex, decreases as compared with normal cholesterol content. Thus the cholesterol increase lags behind the very considerable enlargement of the cortex.

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THE LATENT PERIOD OF CO-CARCINOGENESIS.

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If a chemical carcinogen is applied to the skin of mice at a concentration just too low to produce tumours by itself and, after an interval, a co-carcinogen is repeatedly applied to the same site, tumours appear after a latent period. The length of this period is independent of the length of the interval if the other conditions are kept constant (Berenblum and Shubik, 1949).

When considering what may be happening in the treated tissue during this latent period, it is relevant to ask whether the action of a co-carcinogen such as croton oil produces a sudden irreversible change which progresses to tumour formation without further treatment, or whether the change is gradual and requires uninterrupted treatment; and, if the latter is the case, whether the process is reversible during a part or the whole of its course.

If co-carcinogenesis is the result of a sudden, irreversible, independently progressive change, a single short co-carcinogenic stimulus, if powerful enough, would be expected to yield some tumours, and these should appear as rapidly as those which follow repeated applications.

Effect of single applications of croton oil.

Three groups of stock albino mice of a strain known to be highly susceptible to the action of carcinogens and of croton oil on the skin (Strain S, Salaman and Gwynn, 1951), each of 14 (7 ♂, 7 ♀) young adults, received a single application of 9:10-dimethyl-1:2-benzanthracene (DMBA), followed, after 6 weeks' interval, by single applications of 3 different concentrations of croton oil. Details are given in Table I.

TABLE I.—*Effect of a Single Application of Croton Oil to Mouse Skin 6 weeks after a Single Application of a Carcinogen.*

Primary treatment:

0·3 ml. 0·15 per cent 9:10 dimethyl-1:2-benzanthracene in acetone.

Interval: 6 weeks.

Secondary Treatments:

Group 1.	0·3 ml.	5	per cent	croton oil	in	light liquid paraffin.
Group 2.	,,	2·5	,,	,,	,,	,,
Group 3.	,,	1·25	,,	,,	,,	,,

7 ♂ and 7 ♀ in each group.

The highest concentration of croton oil in light liquid paraffin which could be applied once to these mice without excessive damage to the skin was 5 per cent. Two tumours appeared in Group 2, 80 days after croton oil treatment. This incidence was no greater than in a control group treated with the carcinogen alone.

One may conclude from this result that co-carcinogenesis by croton oil is not to be explained as a sudden irreversible change, independent of subsequent treatment. It is evident that more than one application of croton oil is needed for co-carcinogenesis.

The effect of stopping the applications before the full number of tumours had appeared, and of resuming them later, was next examined.

Effect of intermittent application of croton oil.

Two groups of mice, similar to the above, were treated as shown in Table II.

TABLE II.—*Comparison of the Effects of Continuous and Intermittent Weekly Applications of Croton Oil to Mouse Skin, 6 weeks after a Single Application of a Carcinogen.*

Primary treatment :

0·3 ml. 0·15 per cent. 9:10dimethyl-1:2-benzanthracene in acetone.

Interval : 6 weeks.

Secondary treatments :

Group 4. Weekly applications 0·3 ml. 2·5 per cent croton oil in light liquid paraffin, from 7th week onwards.

Group 5. Weekly applications of the same, from 7th to 13th week, and again from 22nd to 34th week.

7 ♂ and 7 ♀ mice in each group.

(Group 4 originally consisted of 8 ♂ and 8 ♀ mice. One male produced one tumour at the 10th and died at the 12th week, and one female produced no tumours during the period of observation. These two mice were excluded from the records. This exclusion can be regarded as a selection of those mice which produced two or more tumours during the 45 weeks of the experiment.)

The incidence of tumours is illustrated in Fig. 1. Weekly applications of 2·5 per cent croton oil in paraffin to both groups were begun 6 weeks after an initial treatment with 0·15 per cent DMBA. Croton oil treatment of Group 5 was stopped after 8 applications, when the average tumour incidence in both groups was about 0·5 per mouse. Incidence in the two groups increased for a further 2 to 3 weeks, after which incidence in Group 4 continued to rise, following the curve usual in this type of experiment, while that in Group 5 remained almost constant at about 2 tumours per mouse. After 9 weeks without treatment there had been no appreciable change in tumour number in this group, though the tumours already visible had continued to grow. Weekly croton oil applications were then resumed. For a further 7 weeks no increase in number of tumours was observed ; then a steep rise began, similar in slope to that in Group 4, 14 weeks earlier.

When the second course of croton oil treatment had been continued for 12 weeks it was stopped. Tumour incidence in Group 5 had, by then, reached about 11 tumours per mouse; it continued to rise for a short time, as before, then fell a little, and again remained approximately constant, at between 9 and 10 tumours per mouse.

SIGNIFICANCE OF THE RESULTS.

In assessing the significance of these results, it should be noted that the variation between mice in rate of tumour production by the above treatment, as

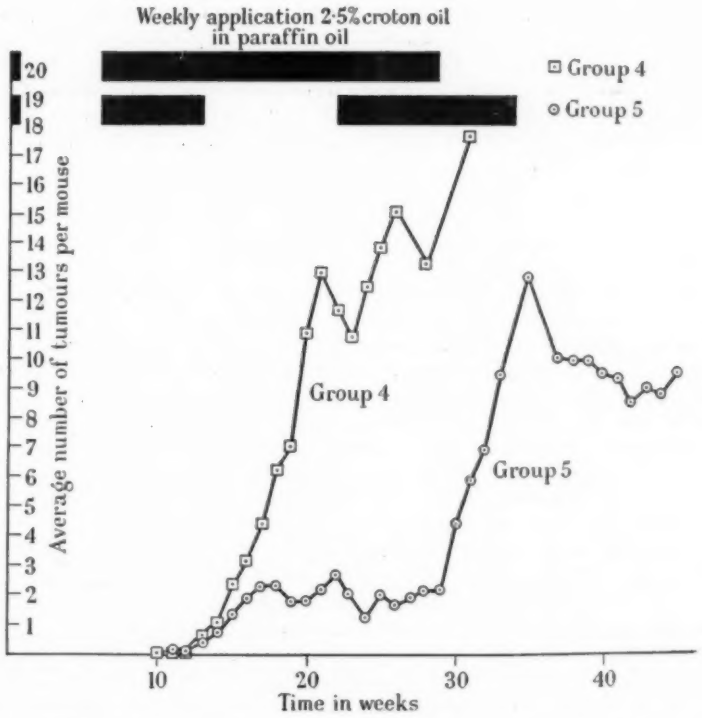

FIG. 1.—Comparison of the effects of continuous and intermittent weekly applications of croton oil to mouse skin, 6 weeks after a single application of a carcinogen.

by others, is high. Consequently individual points in Fig. 1, which represent average numbers of tumours per mouse, have high standard errors, and the differences between corresponding points on the curves of Groups 4 and 5 are barely significant. It is easy to show, however, that the curves as a whole differ significantly. Their slopes between the 15th and 21st week were compared as follows: the average increments of tumour number of individual mice in the two groups from the 15th to the 21st week were 10·64 for Group 4 and 0·93 for

Group 5. The difference between these averages is highly significant ($P < 0{\cdot}001$). The slope of the curve of Group 5 from the 15th to the 29th week was compared with its slope during the succeeding 6 weeks by a similar method. It was shown that the change of direction between these periods is very unlikely to be due to chance ($P < 0{\cdot}001$). Finally, the accuracy with which changes in slope of the curves of tumour incidence can be fixed in time was estimated as follows: The times taken for the appearance of 2 tumours on each mouse of Group 4, reckoned from the beginning of croton oil treatment at the 7th week, and of two new tumours on each mouse of Group 5, reckoned from the beginning of the second course of croton oil treatment at the 22nd week, respectively, were obtained from the records, and the means, and standard errors of the means, of these times calculated. It was found that the first of these mean times was 68 (standard error 4·4) days, the second 58 (standard error 5·5) days. The standard error of the difference between these means is 7·0 days; the observed difference between them of 10 days is therefore not significant. Thus the response to the second course of croton oil treatment was not appreciably more rapid than that to the first.

DISCUSSION.

When croton oil treatment was stopped for the first time, in addition to the few visible tumours there must have been many others of less-than-visible size which would have become visible later if croton oil treatment had continued. A few did appear—one to two per mouse—but that was all. One must suppose that there is a critical size, rather less than 1 mm. diameter; i.e., that tumours smaller than this do not continue to grow without further stimulation. But did these invisible tumours remain unchanged during the remission of treatment? Apparently not, for when the treatment was resumed there was no immediate outburst of newly-visible tumours. After 58 days the interrupted rise in numbers continued. This latent period is not significantly different from that of the response to the first course of croton oil treatment. The skin behaved as it would have done if it had had no previous co-carcinogenic stimulation.

It is possible to conclude that the change produced by croton oil in mouse skin previously treated with a carcinogen is not sudden, or independently progressive. It is a gradual change, requring repeated applications for its maintenance and progress, which is reversible during the greater part of its course, but becomes irreversible not long before tumours become visible.

It has been previously noted (Shubik, 1950*a*, 1950*b*; Salaman and Gwynn, 1951) that of the tumours produced in mouse skin by croton oil following a single treatment with a carcinogen most are benign, though some reveal or develop a malignant character later. The development of malignancy has been excluded from the present enquiry.

SUMMARY.

(1) Single applications of the maximum tolerated dose of croton oil to the skin of mice previously treated once with 9:10-dimethyl-1:2-benzanthracene produced no tumours.

(2) When weekly applications of a well-tolerated dose of croton oil were made to skin previously treated with the carcinogen, tumour production began after a

latent period (defined as the average time taken for 2 tumours per mouse to appear) of 68 days, and continued for 2 to 3 weeks after the treatments were interrupted. When they were resumed after 9 weeks' interval, new tumours began to appear after a latent period (defined similarly) of 58 days. The difference between these two latent periods is not significant.

(3) These results are discussed. It is concluded that the change produced by croton oil in mouse skin previously treated with a carcinogen is a gradual one, requiring repeated treatments for progression towards tumour formation, that it is reversible during the greater part of its course, but becomes irreversible not long before tumours become visible.

I am indebted to Professor S. P. Bedson, F.R.S., for his interest and advice, to Dr. P. Armitage of the Medical Research Council Statistical Research Unit for statistical treatment of the results, and to Miss D. Connell for skilled technical assistance.

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CATALASE POISONS IN RELATION TO CHANGES IN RADIOSENSITIVITY.

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SOME of the effects of ionising radiations are associated with formation of free hydroxyl radicals and possibly of peroxides. Such effects are often reduced under anaerobic conditions and increased in the presence of oxygen. For these reasons it appeared possible that conditions which would increase the concentration of hydrogen peroxide in tissues might augment the biological effects of radiation. Of the possible ways of increasing radiosensitivity by such a mechanism, two have been investigated.

The first depends upon increasing the concentration of substrates, the metabolism of which is known to produce hydrogen peroxide. Such substrates are those oxidised by flavine enzymes, including aldehydes, xanthine, hypoxanthine and *d*-amino acids. This method is being investigated by administration of suitable substrates and riboflavin, the latter in attempts to increase the concentration of flavin enzymes of the tumours.

A second method of increasing hydrogen peroxide concentration of tissues depends upon the inhibition of the enzymes involved in hydrogen peroxide destruction. Thus by inhibition of catalase and peroxidase of tissues this destruction and utilisation of hydrogen peroxide might be prevented and its concentration in cells should increase. This was attempted without success in the present paper.

Many of the known catalase inhibitors including sodium azide and cyanide are also respiratory poisons. When such substances are administered to animals in large doses, they reduce the sensitivity of animals to X-rays (Bacq, 1950). Catalase is, however, much more sensitive than respiration to poisoning by azide, and it should be possible to obtain considerable inhibition of catalase without reducing the respiration. Under such conditions it might be possible to obtain increased radiosensitivity.

An effect of increased sensitivity in tumours, as compared with other tissues, might be obtained by inhibition of catalase because the catalase content of tumours is extremely low, being about one-hundredth of that of liver tissue. Although the amount of catalase present in tumours seems very great when measured at the usual (M/200) concentration of H_2O_2, the activity at naturally occurring concentrations of hydrogen peroxide may be of the same order as the respiration of the tissue, because catalase activity is proportional to the concentration of peroxide.

If the catalase of tissues is measured by disappearance of peroxide, then the measurements might be expected to represent the sum of the catalytic and

peroxidative action of catalase and peroxidase in the tissue, particularly in the case of tumour tissue, in which the apparent catalase activity is low so that the tissue is not diluted much for the determinations. Under such conditions the tissue might be expected to provide substrates for the oxidative action of the hydrogen peroxide.

Catalase activity is often expressed as " Katalasefähigkeit " (Kat.f.), but it can also be expressed by the conventional metabolic expression of Q_{O_2} representing the μl. O_2 liberated per mg. dry weight per hour. Herbert and Pinsent (1948) pointed out that the catalase Q_{O_2} was related to the Kat.f.

$$\left(= \frac{\text{Ko}}{\text{g. preparation in 50 ml. reaction mixture}}\right),$$

so that $Q_{O_2}{}^{H_2O_2} = \text{Kat.f.} \times 77{,}500\ \text{So}$, where Ko = the reaction constant at zero time expressed in logarithms to base 10 and So = the H_2O_2 concentration at zero time. The Q_{O_2} therefore has the same numerical value at the Kat.f. when the concentration of H_2O_2 is M $\times$ 1/77,500 or $1{\cdot}29 \times 10^{-5}$ M, i.e. Kat.f. = $Q_{O_2}{}^{1{\cdot}29 \times 10^{-5}\ \text{M}\ H_2O_2}$. This expression makes it easier to relate the catalase activity of a tissue to the other metabolic processes expressed in Q values. In the tables of this catalase activity is expressed as $Q_{O_2}{}^{1{\cdot}29 \times 10^{-5}\ H_2O_2}$.

The catalase activity is determined at 0° C. because the catalase activity is destroyed rapidly by M/200 H_2O_2 at higher temperatures. Other metabolic processes of mammalian tissues are usually measured at 38°. Presumably catalase in tissues is not destroyed at 38° by H_2O_2 at the naturally occurring low concentrations. The data on the effect of temperature on catalase are difficult to interpret on account of the destruction of the enzyme at higher temperatures. If the activity increased 12·9 fold (which is practically equivalent to a Q_{10} of of 2·0) on increase of temperature from 0° to 38°, then the $Q_{O_2}{}^{10^{-6}\ \text{M}\ H_2O_2}$ measured at 38° would have the same numerical value as the Kat.f. measured at 0°. Thus the Kat.f. value is probably of the same order as the catalase measured as μl. O_2 liberated per hour per mg. dry weight in μM . H_2O_2 at 38°.

Now the figures Kat.f. or $Q_{O_2}{}^{1{\cdot}29 \times 10^{-5}\ \text{M}\ H_2O_2}$ for the Jensen rat sarcoma and the Walker carcinoma lie between 1 and 2 (see Tables VII and VIII) as compared with 100 to 200 for normal rat liver. This means that at 38° the catalase $Q_{O_2}{}^{10^{-6}\ \text{M}\ H_2O_2}$ will be about 2 while the respiratory Q_{O_2} is about 10. Of the respiration of tumours only about 10 per cent is cyanide insensitive (Crabtree and Cramer, 1933), and therefore of the type involving flavine enzymes and presumably likely to be connected with hydrogen peroxide formation. Thus if the basic hydrogen peroxide concentration of the tissue was 10^{-6} M, the rate of decomposition of hydrogen peroxide by catalase would be of the same order as the rate of its formation. With liver tissue this equilibrium might be established with M $\times$ 10^{-8} M H_2O_2 as the cyanide insensitive respiration is of the order of $Q_{O_2} = 2$ and the catalase activity is about a hundred times greater.

If the same hypothesis is extended to tumour tissue in which catalase is poisoned without poisoning of respiration, then with 90 per cent poisoning of catalase the hydrogen peroxide might rise to 10^{-4} M. Such a concentration of peroxide might be expected to increase radiosensitivity if the peroxide were in the cell nucleus and not confined to the cytoplasm.

One of the weaknesses of the foregoing argument is that it involves extrapolation from data obtained with low concentrations of catalase and high concentrations of hydrogen peroxide to the tissue where there is a high concentration of the enzyme and only small amounts of the substrate.

Blaschko (1935) compared the effect of a concentration of hydroxylamine, just sufficient to poison catalase, on the respiration of kidney and of testis. The respiration of kidney began to fall in a few minutes while the respiration of the testis fell much more slowly. The kidney respiration probably involved oxidation of substrates which might be coupled with production of H_2O_2 and so damage the tissue. The testis probably oxidised primary carbohydrate.

Recent work has shown that ionising radiations (Taylor, Greenstein and Hollaender, 1947) and some radiomimetic substances (Butler, and Smith, 1950) induce depolymerization of deoxyribonucleic acid. Some of the biological effects of radiation may be due to such an action occurring in the cell. In the case of ionising radiations the effect is probably due to free hydroxyl radicals which are produced in irradiated water. If increase in hydrogen peroxide concentration should increase sensitivity to X-rays, then addition of hydrogen peroxide to a solution of deoxyribonucleic acid should augment the depolymerising action of X-rays. Such an effect has indeed been found by Conway and Butler (1952).

A large number of substances have been reported as catalase poisons, and some of these are listed in Table I in order of potency. In the present work the activity of catalase poisons is compared with their toxicity to mice and an attempt made to measure the inhibition *in vivo*. The effect of dosing tumour-bearing rats with small doses of sodium azide on the radiosensitivity of the tumours has been examined, and the effect of larger doses in decreasing radiosensitivity observed by Bacq (1951) has been confirmed.

EXPERIMENTAL.

The catalase used for inhibition experiments was a commercial preparation (lot 490309 from Messrs. Armour Laboratories, Chicago, Illinois), used at 0·0033 per cent final concentration in N/100 H_2O_2.

TABLE I.—*Comparison of Inhibition of Catalase and Toxicity of Some Catalase Poisons.*

	Concentration of poison causing inhibition of catalase.		Toxicity for mice. LD 50 mg./kg.	Mol. weight.	LD 50 (in molarity).	LD 50 (in molarity). / Conc. for 50% catalase inhibition.
	50%	90%				
Sodium azide	4×10^{-7}	5×10^{-6}	27	65	4×10^{-4}	1000
Hydroxylamine	2×10^{-6}	2×10^{-5}	175	69·5	4×10^{-4}	500
Cysteine hydrochloride	5×10^{-4}	4×10^{-3}	3000	158	2×10^{-2}	40
p-Methylaminophenol	10^{-5}	7×10^{-4}	40	344	10^{-4}	10
Sodium formate	4×10^{-2}	—	2500	68	4×10^{-2}	10
Hydrazine	2×10^{-4}	6×10^{-4}	200	144	$1·3 \times 10^{-3}$	6
o-Methylhydroxylamine	8×10^{-4}	10^{-2}	300	83·5	$3·5 \times 10^{-3}$	4
Diethyl-*p*-phenylenediamine	6×10^{-5}	4×10^{-4}	35	201	$1·7 \times 10^{-4}$	3
p-Cresol	7×10^{-4}	10^{-2}	160	108	$1·4 \times 10^{-3}$	2
Hydroquinone	8×10^{-4}	—	150	110	$1·3 \times 10^{-3}$	2

The data for inhibition of technical catalase given in Tables I and II and Fig. 1 were found by measurement of the reduction in hydrogen peroxide destruction caused by the enzyme preparation at pH 6·8 in one minute at 0° C.

Tissue catalase was estimated in homogenates made by grinding the tissue with water and carrying out the determinations with a dilution such that the rate of hydrogen peroxide disappearance could be easily followed and K_O was between 0·02 and 0·05.

TABLE II.—*Catalase Poisons.*

Substance.	Conc. causing 50% inhibition.	Reference.
Sodium azide	4×10^{-7}	Blaschko (1935*a*).
Sodium sulphide	8×10^{-6}	Stern (1932).
Potassium cyanide	6×10^{-6}	Rona, Fiegel and Nakahara (1925).
Hydroxylamine	2×10^{-6}	Blaschko (1935*a*).
Dimethyl-*p*-phenylenediamine hydrochloride	5×10^{-5}	Horner and Betzel (1950).
Resorcinol	5×10^{-5}	Blaschko (1935*a*).
Tetramethyl-*p*-phenylenediamine	5×10^{-5}	Horner and Betzel (1950).
p-Hydroxyphenyl azide	2×10^{-5}	This paper.
Mercuric chloride	10^{-5}	Blaschko (1935*a*).
Phenyl hydroxylamine	10^{-5}	Seide (1941).
p-Methylaminophenol	10^{-5}	This paper.
Hydroquinone	8×10^{-4}	,, ,,
o-Methylhydroxylamine hydrochloride	8×10^{-4}	This paper.
p-Cresol	7×10^{-4}	—
Benzidine	5×10^{-4}	Blaschko (1935).
m-Phenylenediamine	5×10^{-4}	,, ,,
Potassium perchlorate	$> 10^{-4}$	,, ,,
Phenyl hydrazine	10^{-4}	,, ,,
Ethyl-hydrogen peroxide	10^{-4}	,, ,,
Sodium hyponitrite	10^{-4}	This paper.
o-Phenylenediamine	10^{-4}	Horner and Betzel (1950).
Pyridinium-aceto-hydrazide chloride	$7{\cdot}5 \times 10^{-3}$	This paper.
Trimethyl-ammonium-aceto-hydrazide chloride	$7{\cdot}5 \times 10^{-3}$	,,
Ethylenediamine	$7{\cdot}5 \times 10^{-3}$	,,
Sulphanilamide	5×10^{-3}	Scholer and Meier (1944).
Sulphapyridine	3×10^{-3}	,, ,, ,,
Cysteine hydrochloride	3×10^{-3}	Stern (1932).
Methylamine	$2{\cdot}5 \times 10^{-3}$	This paper.
Hydrazine hydrochloride	2×10^{-3}	Blaschko (1935).
Semicarbazide hydrochloride	$1{\cdot}5 \times 10^{-3}$	This paper.
Sodium formate pH 5·2	10^{-3}	Agner and Theorell (1946).
Potassium chlorate	10^{-3}	Blaschko (1935).
p-Phenylenediamine	10^{-3}	,, ,,
Cobalt nitrate	10^{-3}	Euler and Glaser (1950).
Sodium cyanate	10^{-3}	This paper
tert.-Butyl hydroperoxide	7×10^{-2}	,,
Acetaldehyde	3×10^{-2}	Stern (1932).
2-Hydroxy-ethylamine	$2{\cdot}5 \times 10^{-2}$	This paper.
Sodium nitrite	2×10^{-2}	,,
Formaldehyde	2×10^{-2}	Stern (1932).
Sodium acetate pH 5·2	10^{-2}	Agner and Theorell (1946).

Catalase activity of tissues was measured by a modification of the method of von Euler and Josephson (1927), in which the fall in concentration of H_2O_2 at pH 6·8 and 0° C. was measured. Aliquots (5 ml.) of the reaction mixture were taken at 3, 5, 7 and 9 minutes and the reaction stopped with 5 ml. 2N H_2SO_4. The residual H_2O_2 was titrated with N/20 $Na_2S_2O_3$ in the presence of 0·01 M ammonium molybdate and 0·1 M KI. The activity of the catalase was expressed

as the Kat.f. value (in Table VI) and as the equivalent amount of O_2 which would be liberated from M/200 H_2O_2 per hour per mg. dry weight of tissue divided by 387. This latter is equivalent to a $QO_2^{H_2O_2}$ value, expressed as $QO_2^{1.29 \times 10^{-4}\ H_2O_2}$ makes the rate of catalase activity comparable with other measurements of metabolism such as QO_2, and has the same numerical value as the Kat.f. value.

Inhibition of catalase in vitro.

The inhibition of catalase activity by different concentrations of known catalase poisons at pH 6·8 and 0° C. is shown in Fig. 1 and the concentrations

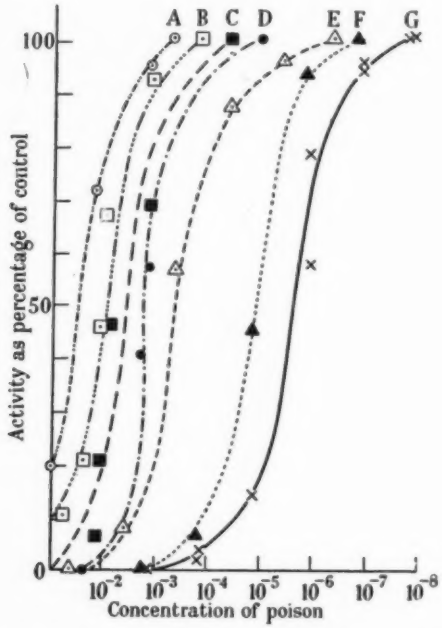

FIG. 1.—Activity of catalase in the presence of different concentrations of poisons.

A: Sodium formate. B: o-Methylhydroxylamine hydrochloride. C: Cysteine hydrochloride. D: Hydrazine hydrochloride. E: Diethyl-p-phenylene-diamine-hydrochloride. F: Hydroxylamine-hydrochloride. G.: Sodium azide.

causing 50 per cent and 90 per cent inhibition are listed in Table I. The toxicities are also listed for some of the compounds, and the ratio of the concentrations causing inhibition of the enzyme to the toxicity expressed as the LD 50 (calculated on a molar basis) is given in Table I. A number of catalase poisons are listed in Table II, and some substances which caused less than 50 per cent inhibition of catalase at the concentrations used are given in Table III.

The most active catalase poison is sodium azide, which was shown to be a catalase poison by Keilin and Hartree (1934); Keilin (1936) and later, Hollinger,

TABLE III.—*Substances with Negligible Catalase Poisoning Action.*

Compound.	Final concentration in molarity.	Poisoning activity.	L.D. 50 for mice (mg./kg.).
1-Nitroso-2-naphthol	$7 \cdot 3 \times 10^{-6}$	25	400
Nitrosoresorcinol	10^{-6}	20	250
2-Nitroso-1-naphthol	$7 \cdot 3 \times 10^{-6}$	12	—
p-Nitrosophenol	$1 \cdot 1 \times 10^{-5}$	5	—
Sulphathiazole	$4 \cdot 7 \times 10^{-4}$	0	—
Urethane	$1 \cdot 4 \times 10^{-2}$	0	2000
Thiourea	$4 \cdot 3 \times 10^{-3}$	0	—
Ethyleneimine	$1 \cdot 5 \times 10^{-1}$	0	15
Tetralin hydroperoxide	$7 \cdot 9 \times 10^{-5}$	0	250
1:3 Dimethylsulphonoxy propane	$6 \cdot 9 \times 10^{-5}$	0	—
1:4 „ butane	$6 \cdot 5 \times 10^{-6}$	0	46
1:8 „ octane	$2 \cdot 1 \times 10^{-6}$	0	7300
Methyl sulphonoxy butane	$1 \cdot 1 \times 10^{-4}$	0	450
Choline chloride	$4 \cdot 3 \times 10^{-2}$	0	—
Trimethylamine-oxide hydrochloride	$6 \cdot 9 \times 10^{-3}$	25	—
Guanidine hydrochloride	$3 \cdot 5 \times 10^{-2}$	0	—
Glyoxal	$2 \cdot 2 \times 10^{-2}$	0	—
Sodium bicarbonate	$5 \cdot 2 \times 10^{-3}$	0	—
Formamide	$1 \cdot 8 \times 10^{-1}$	0	—
Urea	$5 \cdot 5 \times 10^{-2}$	0	—
Nitromethane	$1 \cdot 2 \times 10^{-1}$	28	—
Glycine	$1 \cdot 2 \times 10^{-2}$	0	—
Ethylene glycol	$1 \cdot 1 \times 10^{-1}$	6	—
Allyl alcohol	10^{-1}	11	—

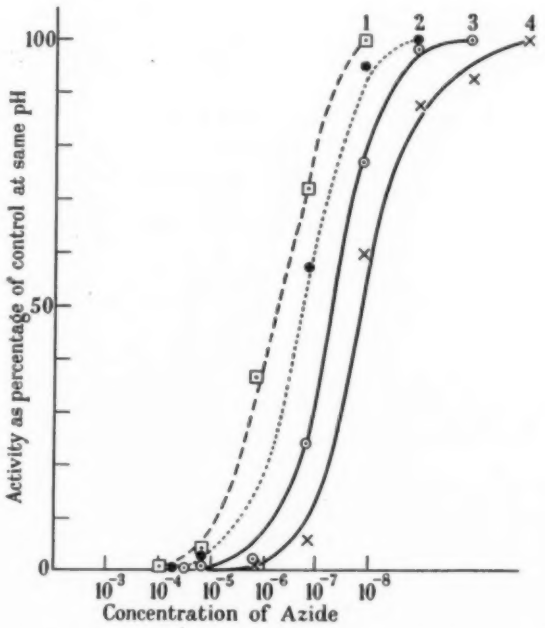

FIG. 2.—Activity of catalase in the presence of sodium azide at different pH values.
Curve 1, pH 7·2; 2, pH 6·8; 3, pH 6·0; 4, pH 5·6.

Fuhrman, Lewis and Field (1949) showed that the inhibition of oxygen uptake of bakers' yeast by 10^{-3} M sodium azide, which is also a respiratory poison, increases on reduction of the pH of the system. The poisoning of catalase by sodium azide was measured at different pH values and is similarly dependent on pH (Fig. 2 and Table IV). The results suggest that the inhibition of respiration and catalase activity are both due to the undissociated hydrazoic acid present

TABLE IV.—*Inhibition of Catalase by Azide at Different Hydrogen Ion Concentrations.*

pH.	Concentration of sodium azide present causing 50% inhibition (Molar × 10^7).	Dissociation of hydrazoic acid (calc.) (%).	Concentration of undissociated hydrazoic acid for 50% inhibition (Molar × 10^9).
5·6	2	93·0	14
6·0	6	96·6	20
6·8	25	99·44	14
7·2	70	99·77	16

TABLE V.—*Poisoning of Catalase by Hydroxylamine and Hydrazine at Different pH Values.*

Hydroxylamine.

Conc. (M).	% inhibition at pH.					
	5·8	6·4	6·8	7·2	7·2	7·8
10^{-4}	67	84	85	86	85	85
10^{-5}	45	56	60	54	50	46
Conc. (M) for 50% inhibition	2×10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}	10^{-5}

Hydrazine.

Conc. (M).	% inhibition at pH.					
	5·8	6·4	6·8	7·2	7·4	7·8
$6{\cdot}8\ 10^{-4}$	69	64	63	55	50	47
$3{\cdot}3\ 10^{-4}$	50	40	34	32	28	19
$1{\cdot}6\ 10^{-4}$	35	26	22	18	12	8
Conc. (M) for 50% inhibition	$3{\cdot}3 \times 10^{-4}$	5×10^{-4}	5×10^{-4}	6×10^{-4}	7×10^{-4}	7×10^{-4}

in the solution. This is in agreement with the fact that the molar concentration of undissociated hydrazoic acid which causes 50 per cent inhibition at different hydrogen ion concentrations is almost constant. This result is considered in the discussion.

The poisoning of catalase with hydroxylamine and hydrazine at different hydrogen concentrations was measured with results shown in Table V. With these basic inhibitors the poisoning appeared to be almost independent of hydrogen ion concentration.

Catalase activity of rat liver and tumour.

Figures for the activity of normal rat liver and tumour and liver of cancerous rats expressed in different notations are given in Tables VI, VII and VIII.

TABLE VI.—*Catalase Activity of Normal Rat Liver.*

Sex.	Weight of rat (g.).	Kat.f value or $QO_2^{1.29} \times 10^{-5}$ M H_2O_2.	$QO_2^{0.5} \times 10^{-2}$ M H_2O_2.
♂	160	170	66,000
♂	145	240	93,000
♂	150	190	73,600
♀	150	78	46,500
♀	152	120	54,300
♀	146	150	62,000

TABLE VII.—*Catalase Activity in Jensen Sarcoma-bearing Male Rats.*

Weight of rat (g.).	Days from implantation.	$QO_2^{1.29} \times 10^{-5}$ H_2O_2 or Kat.f.	
		Tumour.	Liver.
260	15	0·87	67
264	16	0·95	88
280	16	1·42	112
240	19	1·23	96

TABLE VIII.—*Inhibition of Catalase Activity after Injection of Catalase Poisons into Walker Carcinomata-bearing Male Rats.*

Days from implantation of tumour.	Dose mg. per kg. body-weight.	Poison injected.		Kat.f. or $QO_2^{1.29} \times 10^{-5}$ H_2O_2.	
				Tumour.	Liver.
7	—	None		1·9	35·3
8	—	—		1·3	24·7
8	—	—		1·2	13·4
6	—	—		1·8	48·7
6	—	—		2·0	62·7
6	—	—		1·4	68·3
6	—	—		0·89	56·2
7	125	Hydroxylamine	5 min. before	0·82	16·2
6	50	,,	15 ,, ,,	1·8	27·4
10	25	,,	15 ,, ,,	0·85	27·9
8	20	,,	15 ,, ,,	1·7	33·5
8	20	,,	15 ,, ,,	2·2	46·7
10	10	,,	15 ,, ,,	0·89	58·2
7	15	Sodium azide	15 ,, ,,	0·1	8·8
7	15	,,	15 ,, ,,	0·2	12·8
10	15	,,	15 ,, ,,	0	5·8
13	15	,,	4 hrs. before	—	31·9
12	15	,,	18 ,, ,,	2·7	17·5
17	7·5	,,	15 ,, ,,	0·8	7·6
17	1·5	,,	15 ,, ,,	1·8	32·8

TABLE IX.—*Catalase Inhibition of Liver* in vivo.

Male rats injected intraperitoneally with different poisons and killed 15 minutes later.

Weight.	Poison injected.	Kat.f. or $QO_2^{1.29} \times 10^{-5}$ M H_2O_2.
150	15 mg./kg. sodium azide	9·3
160	,, ,,	18·9
170	,, ,,	37·8
170	25 mg./kg. hydroxylamine	96·2
168	,, ,,	130·5
170	,, ,,	103·0
158	125 mg./kg. hydrazine	124·5
145	,, ,,	150·0
150	,, ,,	96·5

The poisoning of catalase *in vivo* is difficult to estimate owing to the reversibility of the poisoning. Blaschko (1935*a*) showed that of a number of catalase poisons only potassium chlorate produced irreversible inhibition. The results of Blaschko (1935*a*) and also of Foulkes and Lemberg (1949), however, indicated that the poisoning by azide was less easily reversed than that by hydroxylamine. These findings would account for the results (Tables VIII and IX) of *in vivo* poisonings with these agents. Reduction of the catalase activity of Walker carcinoma tissue was seen following dosage of the rats with sodium azide but not after treatment with hydroxylamine. It is therefore difficult to determine how much enzyme inhibition is produced *in vivo* when agents are injected into animals when the inhibition is reversible as it is with hydroxylamine.

TABLE X.—*The Effect of Pretreatment of Rats on the Radiosensitivity of the Walker Carcinoma.*

The irradiation was given to the tumour only on the fifth day after transplantation.

Exp.	Treatment of groups.			
	A.	B.	C.	D.
1	Control	1200 r.	NH_2OH 20 mg./kg. alone	NH_2OH 20 mg./kg. 15 min. before 1200 r.
2	„	1200 r.	NaN_3 15 mg./kg. alone	NaN_3 15 mg./kg. 15 min. before 1200 r.
3	„	1200 r.	NaN_3 1·5 mg./kg. alone	NaN_3 1·5 mg./kg. 15 min. before 1200 r.
4	„	600 r.	NaN_3 1·5 mg./kg. alone	NaN_3 1·5 mg./kg. before 600 r.
5	„	600 r.	NaN_3 1·5 mg./kg. alone	NaN_3 1·5 mg./kg. before 600 r.

In no case was the difference in growth of tumours between Groups B and D of significance.

The effect of treatment of the Walker carcinoma with X-rays and premedication with catalase poisons.

Four groups each of ten rats were grafted with the Walker carcinoma and the resulting tumours were measured thrice weekly. When the tumours were established (at 5 or 6 days after grafting) the groups of rats were treated as shown in Table X. The pretreatment with the catalase poisons tried did not modify the effect of radiation on the tumour.

Reduction of sensitivity of mice treated with sodium azide and hydroxylamine to X-radiation.

Groups of each stock of 10 mice were exposed to 700 r irradiation from an X-ray tube operated at 220 kVp. at 15 mA. giving rays with 1 mm. copper and 1 mm. aluminium filter, at a rate of 140 r per minute with FSD = 100 cm.

The results (Fig. 3) show that pretreatment with sodium azide or hydroxylamine had a protective action. The result with azide is similar to that obtained by Bacq (1951), but the result with hydroxylamine appears to be a new finding in general agreement with Bacq's work.

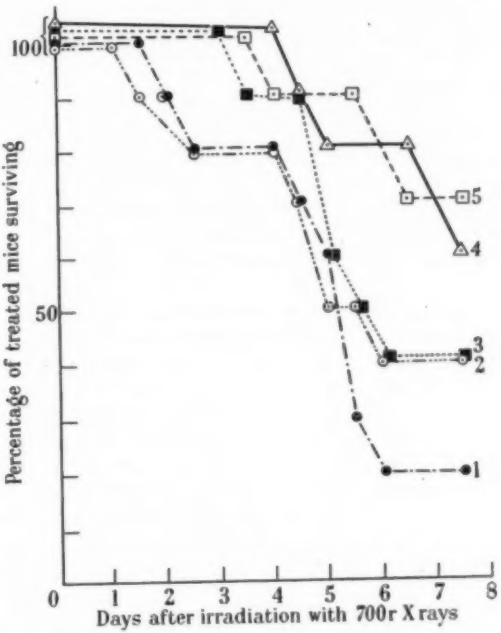

FIG. 3.—Survival of mice after X-irradiation.

1: Control. 2: Injected with 1·5 mg. per kg. NaN_3 15 min. before irradiation. 3: Injected with 15 mg. per kg. NaN_3 15 min. after irradiation. 4: Injected with 25 mg. per kg. hydroxylamine hydrochloride 15 min. before irradiation. 5: Injected with 15 mg. per. kg. NaN_3 15 min: before irradiation.

DISCUSSION.

The attempt to increase sensitivity of tumours to X-rays by poisoning the catalase of grafted tumours *in vivo* with azide or hydroxylamine was unsuccessful. This might be because the amounts of hydrogen peroxide produced in tumour metabolism are too small to have any effect in augmenting the action of radiation. The hydrogen peroxide may, however, be produced and utilised in the tumour tissue. The absence of increased sensitivity is, however, more probably due to the fact that the catalase poisons used are also agents which protect animals from radiation. These substances may combine or neutralise free hydroxyl radicals. For these reasons work should be carried out to measure tumour peroxidases and try to inhibit these as well as catalase, and to find catalase poisons which are not protective agents against radiation.

The search for a poison more specific for catalase than is azide was also unsuccessful. Sodium azide and hydroxylamine are much the most specific catalase poisons of the compounds examined. *p*-Hydroxyphenylazide had only 1/50 of the activity of sodium azide (calculated on a molar basis). This difference is exactly the same as that found between hydroxylamine and phenylhydroxylamine. On the other hand, phenylhydrazine appears to be a more potent inhibitor than hydrazine (Blaschko, 1935*a*). The introduction of a methyl group into the hydroxylamine molecule (o-methyl-hydroxylamine) reduced the activity 400 fold. The inhibition produced by diethyl-*p*-phenylenediamine was of the same order as that described for dimethyl-*p*-phenylenediamine by Horner and Betzel (1950).

The figures on the ratio of catalase inhibiting concentration to lethal dose show that sodium azide is the most favourable catalase poison as well as the most active. The median lethal dose of sodium azide should give a concentration of azide which is one thousand times that causing 50 per cent inhibition of catalase. Thus, sodium azide is probably the safest catalase poison for use *in vivo* in spite of its high toxicity.

The increase in poisoning activity of azide with decrease in the pH is similar to that found for the effect of formate and acetate by Agner and Theorell (1946), who showed that anions in general combine with catalase and inactivate it to an extent increasing with decrease in pH. These authors explained this as due to the anion displacing the hydroxyl group of the catalase and it is possible that azide acts in the same way. The data of Agner and Theorell (1946) on poisoning of catalase with formate at different pH values are analogous to those found for azide (Table IV) as shown in Table XI. The calculated concentration of undissociated formic acid which causes 50 per cent inhibition is seen to be constant for any one temperature and so analogous to our results with azide.

TABLE XI.—*Inhibition of Catalase by Formate at Different pH Values (data from Agner and Theorell* 1946).

pH.	Temp.	Conc. formate for 50% inhibition.	Proportion undissociated (%).	Calculated conc. of undissociated formic acid.
3·37	0·8°	2×10^{-6}	75	$1{\cdot}5 \times 10^{-6}$
5·18	0·8°	6×10^{-5}	2	$1{\cdot}2 \times 10^{-6}$
3·35	20°	5×10^{-6}	75	$3{\cdot}7 \times 10^{-6}$
3·99	20°	10^{-5}	40	4×10^{-6}
5·20	20°	2×10^{-4}	2	4×10^{-6}

These findings may be interpreted as Agner and Theorell (1946) suggest as due to replacement of the OH of the catalase by azide or formate. The fact that the inhibition seems to depend on the concentration of unionised poison suggests that it is the undissociated molecule which combines with the enzyme. Now these effective poisons resemble the substrate in shape. It is established that the atoms of hydrazoic acid are in a chain rather than in a ring, and some other simple catalase poisons are of about the same size and shape as hydrogen peroxide (Table XII). In hydroxylamine one of the oxygen atoms is replaced by an NH group, and in hydrazine both oxygen atoms are so replaced by NH

TABLE XII.—*The Substrate and Poisons of Catalase.*

Hydrogen peroxide	H	O	O	H
Hydrazoic acid	H	N	N	N
Hydroxylamine	H	O	NH	H
o-Methylhydroxylamine	CH_3	O	NH	H
Hydrazine	H	NH	NH	H
Methylamine	H	CH_2	NH	H
Nitrous acid	H	O	N	O
Formic acid	H	O	CO	H
Acetic acid	H	O	CO	CH_3

groups. In formic acid one oxygen atom of hydrogen peroxide is replaced by a CO group. The replacement of a hydrogen of formic acid by CH_3 as in acetic acid reduces the activity 800 fold (Agner and Theorell, 1946), which is the same order as that produced by the introduction of an o-methyl group in hydroxylamine.

Thus the poisons may combine readily with catalase, possibly replacing a hydroxyl group or hydrogen peroxide because of their shape and size. In testing this hypothesis, methylamine was found to be a poison (of the same order as hydrazine), but ethyl formate and formamide, which it was hoped would be as effective as undissociated formic acid, were both found to be inactive.

While the poisoning with hydrazoic acid and formic acid may depend upon the entire undissociated molecule, the poisoning with hydroxylamine and hydrazine appeared to be independent of the pH. Now in the cases of hydrazoic acid and formic acid, ionisation would result in loss of a hydrogen atom (and gain of a charge), so that the ion would have less structural resemblance to hydrogen peroxide. On the other hand, the salts of hydroxylamine and hydrazine have added hydrogen atoms and charge, which presumably makes little difference to the poisoning action. These results show that although the shape of the molecule may be important, it is not the only factor controlling catalase inhibition for this type of poison.

Catalase poisons may be divided into at least three groups :

(a) Those combining with iron, such as cyanide and sulphide.

(b) Those combining with the enzyme possibly because of a formal resemblance to the substrate such as azide and hydroxylamine.

(*c*) Those which act as substrates for peroxidase such as *p*-phenylenediamine and hydroquinone (Stern and Bird, 1951).

SUMMARY.

(1) A list of catalase poisons including some hitherto unknown poisons is given, and the concentration required for 50 per cent inhibition of catalase is compared with the lethal dose for mice. Azide and hydroxylamine are the most specific of known catalase poisons.

(2) The poisoning with azide increases with decrease in pH so that the concentration of undissociated hydrazoic acid required for 50 per cent inhibition remains constant. The poisoning by hydroxylamine and hydrazine is almost independent of pH. The poisoning of catalase is considered in relation to these findings that hydrazoic acid and formic acid act as undissociated molecules. The possibility that poisons of this type are effective because of their structural resemblance to hydrogen peroxide is discussed.

(3) Poisoning of catalase of liver and tumour of rats was demonstrated after injection of azide.

(4) Injection of small doses of azide or hydroxylamine into rats immediately before X-irradiation did not increase the radiosensitivity of tumours. Injection of large doses of azide or hydroxylamine into mice before irradiation reduced the mortality from X-irradiation of the whole body.

(5) Catalase activity could be expressed as a $Q_{O_2}^{1.29 \times 10^{-4}\ H_2O_2}$ value in μl. O_2 which would be liberated, per hour, per mg. dry weight of tissue; this has the same numerical value as the Kat.f.

Since this paper was sent for publication the authors have seen the communications of B. Chance (1952) in which the poisoning of catalase by azide and formate is shown to depend on the concentration of undissociated molecules of the acids concerned.

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COMPARISON OF CYTOLOGICAL EFFECTS INDUCED BY X RAYS AND NITROGEN MUSTARD.

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THE possibility that the " mustards " might be of biological interest was first suspected during the last war. At that time research into their pharmacological and toxicological properties showed that certain of their effects were strikingly similar to those of ionizing radiation (Gilman and Philips, 1946 ; Karnofsky, Graef and Smith, 1948 ; Philips, 1950). This led to further investigations of their action as mutagenic agents and as growth inhibitors and their importance in these connections quickly became apparent. On account of the great similarity shown by the effects of mustards to those of irradiation the mustards were labelled as " radiomimetic poisons " (Dustin, 1947).

So far as their mutagenic effects are concerned, these compounds have been found to be highly active. Like X rays they are able to produce genetic changes at dose levels far below those at which any toxicological change can be demonstrated by present biochemical techniques. However, the types of genetic change which they induce are in some respects different from those caused by X rays, and evidence is now accumulating which strongly suggests that the mode of action of the two agents is also very different (Auerbach, 1950).

During the present investigation the cytological effects induced by X rays and one of the mustards (methyl-bis-(β chloroethyl)amine or HN2) in tumour cells of the transplantable Walker carcinoma 256 have been analysed and compared. These effects, consisting primarily of chromosome fragmentation, were found by Koller (1948) to underlie inhibition of normal and malignant growth in tissues. Our aim was to throw some light on the basic biological reaction which results in temporary or permanent inhibition of growth, and to ascertain if possible in what respects the mode of action of X rays and nitrogen mustard differ in producing their inhibition.

EXPERIMENTAL TECHNIQUE.

Walker carcinoma 256 was employed for all experiments. It was grafted subcutaneously into white albino rats fed *ad libitum* on routine mixed diet containing about 15 per cent protein. Animals of both sexes were used weighing from 150 to 230 g. Tumours were selected for treatment on the 6th or 7th day after implantation.

The standard dose level adapted as the basis for comparison of chemical and X-ray effects was 1 mg. of HN2 per kg. body weight. In preliminary experiments the chromosome effects of this dose in femoral bone marrow and tumour were compared with the effects of 100, 200, 400 and 500 r whole body irradiation. These

analyses showed that 200 r might be considered to be an " equivalent " dose, i.e., it produced in tumour and bone marrow the same amount and the same type of cellular injuries up to 24 hours after administration (Koller, unpublished). The previously reported X-ray " equivalent " dose based on the 50 per cent lethal dose (LD 50) is about 400–500 r.

In the first set of experiments irradiation with 200 r was applied direct to the tumour. The technical conditions were as follows: 140 kV 0·1 mm. Cu filter, focal skin distance of applicator 13 cm., dosage rate 140 r/min. (Elson and Lamerton, 1949). During the investigation it was found that beside the cellular effects HN2 also produced significant histological changes. It was suspected that systemic toxic effects of HN2 might be responsible for this additional effect. In order to study the influence of systemic effects of X rays on cellular reaction in another set of experiments 200 r (and 500 r) was given as whole body irradiation under the following conditions: 220 kV half value layer 1·5 mm. Cu, focal skin distance 90 cm., dosage rate 7·5 r/min.

When it was found that the toxic effects of 200 r whole body irradiation did not increase cellular reaction, tumours were exposed to 300 and 1000 r in order to find if doses higher than 200 r can produce histological changes similar to those observed after HN2 administration. These experiments in which various doses and methods of irradiation are used have been undertaken to demonstrate the widest possible spectrum of the biological reactions which can be brought about by X rays in the Walker carcinoma.

Nitrogen mustard was administered by single intraperitoneal injection using freshly prepared aqueous solution of 0·02 per cent of the commercial compound methyl-bis-(β chloroethyl) amine hydrochloride (Boots & Co.). The dose was 1 mg. per kg. body weight throughout the experiments.

For cytological analysis small pieces of tumours were fixed in acetic alcohol mixture (1 : 3), and squash preparations were made and stained with Feulgen's basic fuchsin. For histological study fragments were fixed in Bouin's solution, embedded in paraffin wax, and sections cut at 5μ were stained with haematoxylin and orange-G.

Cytological effects: method of analysis.

The method used for estimating cellular injuries is the same as was employed by Devik, Elson, Koller and Lamerton (1950). Cells in post-metaphase stages were selected. At this stage the " acentric " fragments can be seen, scattered between the two daughter chromosome groups (Fig. 1, 2). " Dicentrics " are seen as chromosome bridges at this stage and these also were recorded (Fig. 3, 4). The " micronuclei " of resting cells are a further criteria of mitotic injury; they represent chromosome fragments left in the cytoplasm at the completion of mitosis (Fig. 5). None of these abnormalities occur except as an extreme rarity in control tumours; in two control experiments we found only 3 and 5 per cent of cells with any mitotic disturbance and these were mostly cases of stickiness of chromosomes at anaphase. These have, of course, an origin quite different from that of true bridges, brought about by dicentric chromosomes or chromatids (Koller, 1947), and they could be distinguished and classified accordingly.

It should be mentioned that the tumour-bearing animals were unrelated and were treated and killed at varying times. Their genetic heterogeneity is reflected

in the variation shown by the quantitative data. The importance of some biological variables (age, sex, diet, etc.) has already been discussed; Devik and co-workers (1950) demonstrated by analysis that the data, obtained by the same method as used in the present study, do in fact provide a valid basis for comparison. But as will be seen, the effects observed 72 hours after treatment by X ray and HN2 were in any case qualitatively so different that they could not be compared on a quantitative basis.

X-ray effects: localised treatment (200 *r*).

The tumours only were irradiated and cytological effects were analysed at 6, 12, 24, 48, 72, 96, 120, 144 and 168 hours after treatment. The data obtained are presented in Table I. It can be seen that the number of cells with chromosome injuries is highest in the tumour sample which was taken 12 hours after treatment.

TABLE I.—*The Various Cell Injuries Induced in the Walker Carcinoma after* 200*r*.

I.	II.	III.	IV.		V.						VI.	VII.	VIII.	IX.
			No.	%	0.	1.	2.	3.	4.	5+.				
200 r (tumour)	6	50	8	16·0	44	3	3	0	0	0	5	0·18	1·6	5.xii.50
	12	50	14	28·0	39	7	4	0	0	0	5	0·30	8·4	5.xii.50
	24	50	11	22·0	41	7	1	1	0	0	3	0·24	7·2	5.xii.50
	48	50	16	12·0	47	2	0	1	0	0	3	0·10	2·6	7.xii.50
	72	100	5	5·0	97	2	1	0	0	0	2	0·04	3·0	18.v.51
	96	100	5	5·0	97	2	1	0	0	0	2	0·04	1·1	19.v.51
	120	100	3	3·0	98	2	0	0	0	0	1	0·02	0·5	20.v.51
	144	100	2	2·0	98	1	1	0	0	0	0	0·03	0·4	21.v.51
	168	100	(2)*	0·0	0	0	0	0	0	0	0	0	0·5	22.v.51

* Sticky chromosome bridges, probably not due to X ray.

Key to Table I.

I. X-ray dose in roentgens or HN2 dose in mg/kg.
II. Time after treatment in hours.
III. Total number of cells in post-metaphase analysed.
IV. Number and percentage of injured cells in post-metaphase.
V. Number of cells with 0- 5+ fragments.
VI. Number of cells with bridges.
VII. Number of fragments per cell.
VIII. Percentage of cells in resting stage with micronuclei.
IX. Date when the animal was killed.

This is also the sample in which the number of chromosome fragments per cell is at a maximum. The damage is less 24 hours after irradiation, and it diminishes further in the 48 and 72-hour samples. In tumours 4 to 6 days after treatment the number of dividing tumour cells with abnormalities is very small. Our observations indicate that 200 r is insufficient to inhibit the growth of the Walker carcinoma; the growth rate of treated tumours 6 days after irradiation was found to be the same as that of the controls. This finding is in close agreement with that of Devik and co-workers (1950).

HN2-induced cytological effects.

It has already been reported by the senior author that a dose of 1 mg. per kg. body weight of HN2 produces chromosome fragmentation in cells of the Walker

carcinoma (Boyland, Clegg, Koller, Rhoden and Warwick, 1948). In the present experiments the same dose was given, and it was intended to carry out an analysis similar to that of the X-rayed tumours. The type and amount of injury was successfully analysed in the 6, 12, 24 and 48-hour tumour samples (Table IIA), and the comparison of these data with those from X-rayed tumours is fairly straightforward. The total number of tumour cells showing chromosome injuries

TABLE IIA.—*The Various Cell Injuries Induced in the Walker Carcinoma from 6 to 48 hours after HN2 Administration.*

I.	II.	III.	IV.	V.						VI.	VII.	VIII.	IX.
			No.	%.	0.	1.	2.	3.	4.	5+.				
HN2 1 mg. per kg. body weight	6	50	7	14·0	47	2	1	0	0	0	4	0·08	0·8	5.xii.50
	12	50	11	22·0	42	4	3	0	0	1	4	0·40	0·2	5.xii.50
	24	50	16	32·0	34	2	2	0	2	10	9	2·12	2·2	6.xii.50
	48	50	14	28·0	37	2	2	0	2	7	10	2·30	14·8	7.xii.50

Key to Table IIA.—Same as that of Table I.

TABLE IIB.—*The Frequency of Injured Tumour Cells and Histiocytes from 72 to 168 hours after HN2 and 200 r Whole Body Irradiation.*

			Tumour cells.				Histiocytes.				
I.	II.	III.	IV.	V.		VI.	VII.	VIII.		IX.*	X.
				No.	%.			No.	%.		
HN2 1 mg. per kg. body weight	72	100	77	42	54·7	15·9	23	2	8·7	3·3	24.v.51
	96	100	53	48	90·5	19·9	47	5	10·6	1·1	25.v.51
	120	100	34	32	94·0	24·1	66	20	30·3	0·5	14.ix.51
	144	100	42	37	88·0	23·0	58	16	27·4	0·7	15.ix.51
	168	100	18	8	44·4	31·2	82	8	9·7	0·2	16.ix.51
200 r (Whole body)	72	100	88	4	4·5	8·9	12	2	(16·6)	7·3	1.vi.51
	96	100	93	8	8·6	2·9	7	0	—	13·2	2.vi.51
	120	100	83	3	3·6	1·2	17	1	(5·8)	12·4	3.vi.51
	144	100	85	7	8·4	1·2	15	2	(13·3)	5·6	4.vi.51
	168	100	90	2	2·2	1·1	10	0	—	9·0	5.vi.51

* $\frac{\text{Tumour cell}}{\text{Histiocyte}}$ ratio in control tumour is 6·6.

Key to Table IIB.

I. HN2 dose in mg/kg. and 200 r (whole body) treatment.
II. Time after treatment in hours.
III. Total number of cells in post-metaphase stages analysed.
IV. Total number of tumour cells in post-metaphase stages.
V. Number and percentage of injured cells in post-metaphase stages.
VI. Percentage of cells in resting stage with micronuclei.
VII. Total number of histiocytes in post-metaphase stages.
VIII. Number and percentage of injured cells in post-metaphase stages.
IX. Ratio of dividing tumour cell and histiocytes $\frac{(T)}{H}$.
X. Date when the animal was killed.

up to 24 hours after treatment is 34 out of 150 cells analysed. It is the same as was found after irradiation with 200 r (33 out of 150). It can be seen, however, that breaks appear somewhat more slowly after HN2 than after X-ray treatment. The number of cells with HN2-induced injuries increases gradually from 6 to 48 hours; after that period their number increases rapidly. This is the first significant difference which appears between the effects of HN2 and X rays.

However, the analysis 72 hours after treatment and onwards was found increasingly difficult owing to the excessive amount of chromosome injury per cell (Fig. 6, 7) and to a great numerical increase of the undifferentiated histiocytes or fibroblasts. The criteria for the classification of breaks had therefore to be simplified. The dividing cells are grouped into two classes only, normal and abnormal, attributing abnormality to treatment, and tumour cells and histiocytes are scored separately in all samples 72 hours after treatment (Table IIB).

It can be seen that the proportion of dividing tumour cells with chromosome injuries increases up to 144 hours ; but the analysis also shows that tumour cells in mitosis become gradually less frequent and almost disappear 7 days after treatment. Thus out of 100 mitotic cells only 18 were tumour cells, 8 of which were injured. The numerous chromosome bridges in some of these cells strongly suggest that they are undergoing second time mitosis after treatment. By including such cells in the analysis, the true number of injured cells is reduced (compare the 144 and 168 hour samples, Table IIB).

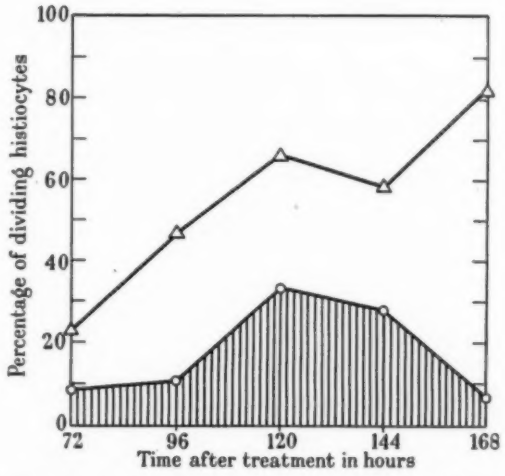


FIG. 9.—The number of dividing histiocytes in the Walker tumour after HN2 administration. The proportion of histiocytes with chromosome injuries is indicated by the shaded area.

It has been mentioned that histiocytes are recorded separately in samples 72 hours after treatment. Histiocytes can be distinguished from tumour cells at mitosis by their much smaller size (Fig. 8). They are particularly evident in young tumour implants and are found in large numbers at the periphery of growing tumours. Although these cells are normally present in the Walker carcinoma, they play a small part in the histological organisation of the tumour. The great and rapid increase in histiocytes after HN2 administration, which takes place in the cellular tumour parenchyma, has important consequences.

Table IIB shows the drastic change which occurs in the cell composition of the tumour. The shift in the proportion of dividing histiocytes and tumour cells is illustrated in Fig. 9. The average level of " tumour cell : histiocyte ratio " in

non-treated tumour is 6·6, indicating that 13·8 per cent of the dividing cell are of the histiocyte type. This figure was derived from analysing 5 control tumours of the same age. The variation is very considerable ; the lowest limit was found to be 5 and the highest 20 per cent.

The histiocytes of treated tumours were seen to have chromosome injuries similar to those of tumour cells. The data indicate a slowing down of the rate of increase of histiocytes between 120 and 144 hours ; i.e., the period during which about 30 per cent of these cells carry injured chromosomes. It is also the period in which the highest damage is recorded for the histiocytes and may be responsible for the slowing-down phenomenon (Fig. 9). It seems that this correspondence can be taken as evidence to show that the increase in the histiocyte count is mainly due to multiplication of these cells, and that migration through the vascular system plays only a minor rôle. Table IIB shows that the relative number of injured histiocytes is less than that of the tumour cells of the same sample, which suggests a difference in the response of the two cell types to nitrogen mustard.

Besides the effects found in dividing cells, definite morphological alterations were also seen in non-dividing cells. Thus the size or volume of many tumour cells increased to about 4 times the normal. In these large cells the nuclear network became coarse and loaded with several heavily stained chromocentres. Often the cytoplasm exhibits various degrees of basophily. Such changes are characteristic of cells undergoing degeneration, and it is probable that these cells are dying. The fact that many tumour cells with these gross morphological changes have no micronuclei suggests that this process of degeneration cannot be attributed to a defective nucleus. It seems more probable that the degeneration is brought about by a non-specific metabolic disturbance in the cytoplasm due directly to HN2.

EXPLANATION OF PLATES.

FIG. 1.—Dividing tumour cell in late anaphase with one acentric chromosome fragment 6 hours after treatment with 200 r. × 2400.

FIG. 2.—Dividing tumour cell in anaphase with acentric chromosome fragment 6 hours after treatment with nitrogen mustard. × 2400.

FIG. 3.—Dividing tumour cell showing chromosome bridge and acentric fragments 24 hours after HN2 administration. × 2400.

FIG. 4.—Chromosome bridge and acentric fragments in tumour cell 24 hours after 1000 r. × 2400.

FIG. 5.—Tumour cells in resting stage after HN2 treatment ; upper cell has one micro-nucleus in the cytoplasm. The altered morphological structure of the nucleus indicates that this cell is undergoing degeneration. × 2400.

FIG. 6.—Dividing tumour cell 72 hours after HN2 administration showing several bridges and fragments. × 2400.

FIG. 7.—Dividing tumour cell 144 hours after HN2 treatment, showing a complete disorganisation of chromosome mechanism. × 2400.

FIG. 8.—Tumour cell (on right) and histiocyte (on left) in anaphase. The tumour cell has a chromosome bridge. × 2400.

FIG. 10.—Walker carcinoma 256, 6 days after implantation. × 340.

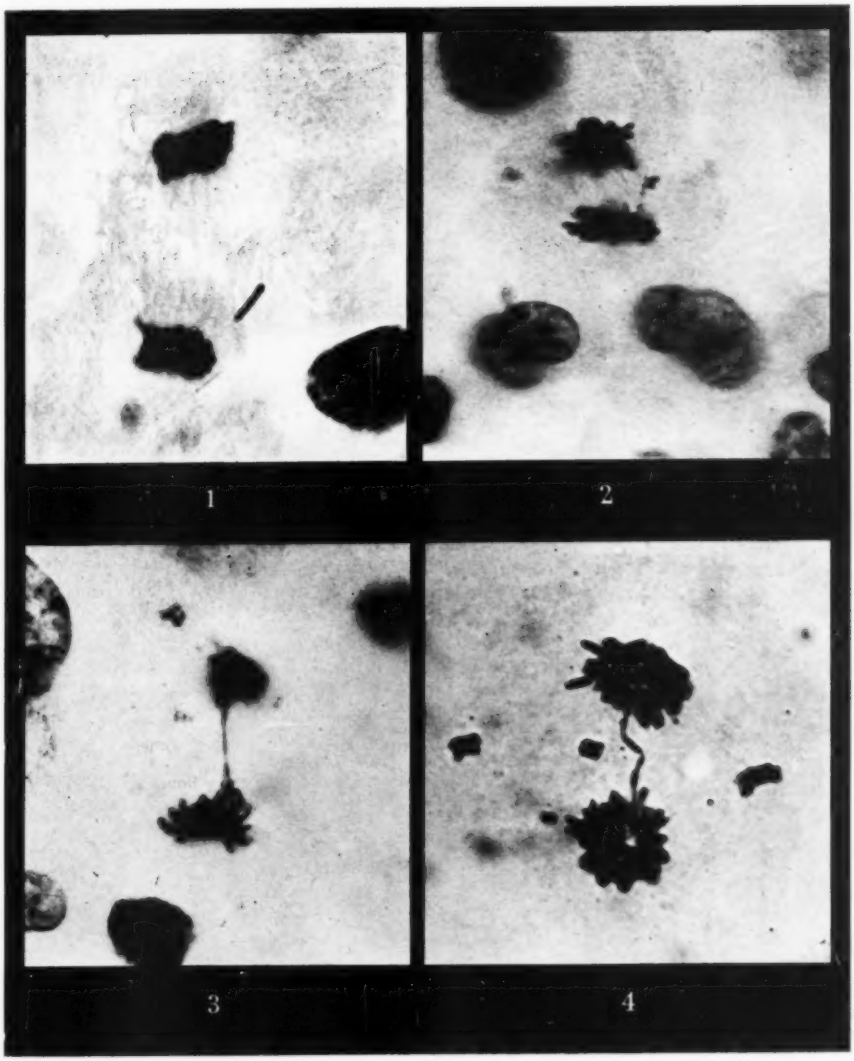
FIG. 11.—Walker carcinoma 72 hours after HN2 administration showing the great increase of histiocytes. × 340.

FIG. 12.—Walker carcinoma 96 hours after HN2 administration. One tumour cell is in mitosis, showing numerous bridges. Tumour cells are scattered and many histiocytes are undergoing mitosis. × 340.

FIG. 13, 14.—Walker carcinoma 96 hours and (Fig. 14) 144 hours after HN2 administration showing fibrosis in the tumour parenchyma. × 340.

FIG. 15.—Walker carcinoma 96 hours after 200 r whole body radiation. × 340.

FIG. 17, 18.—Walker carcinoma 144 hours after 1000 r showing the centre (Fig. 17) and periphery (Fig. 18) of the tumour. × 340.



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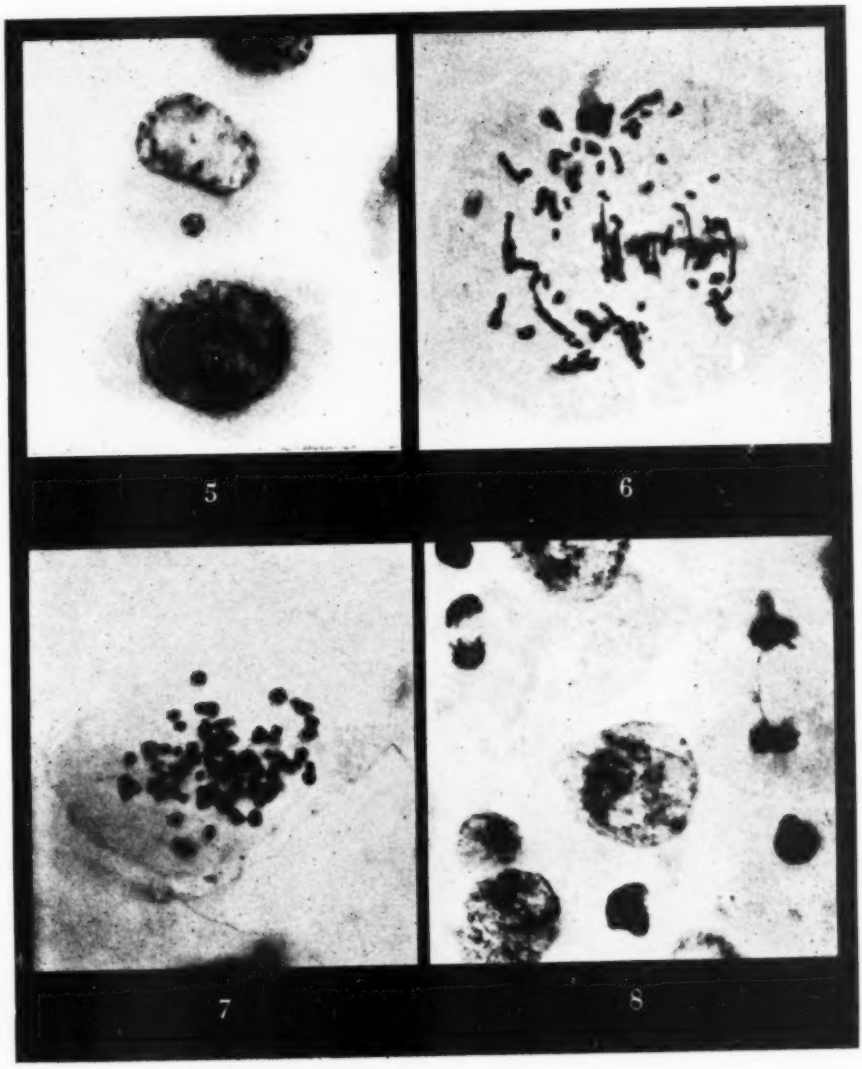

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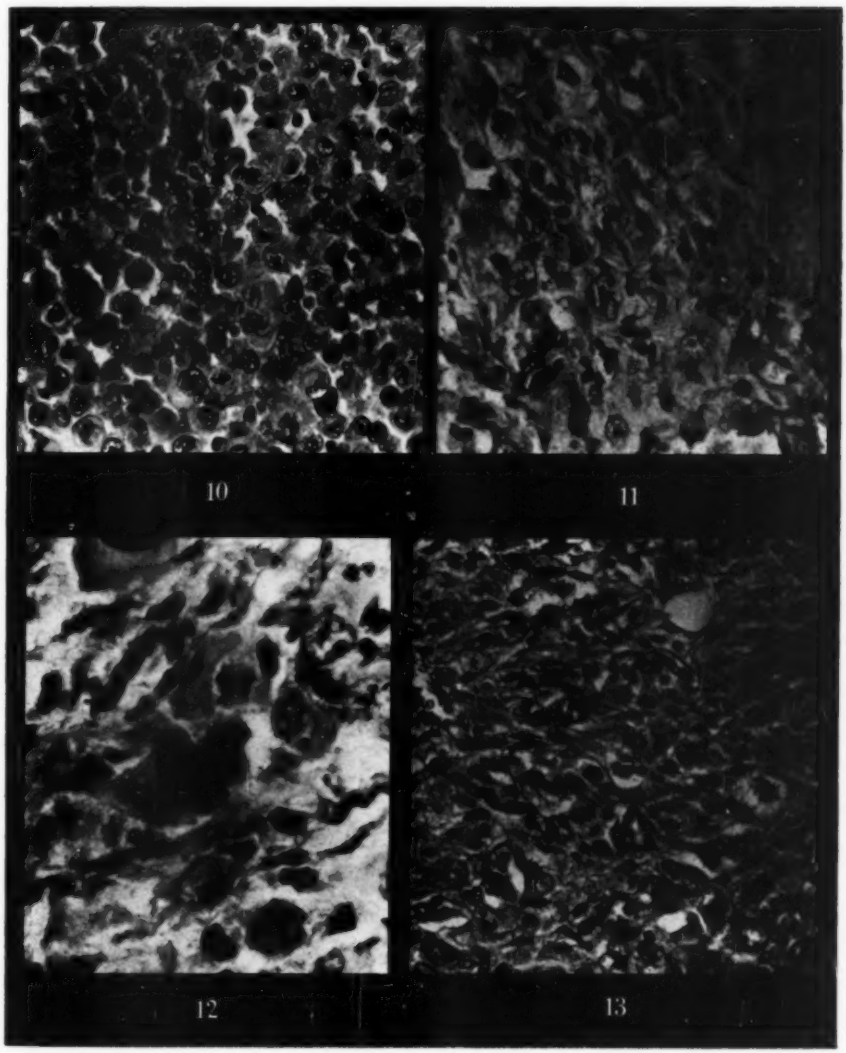

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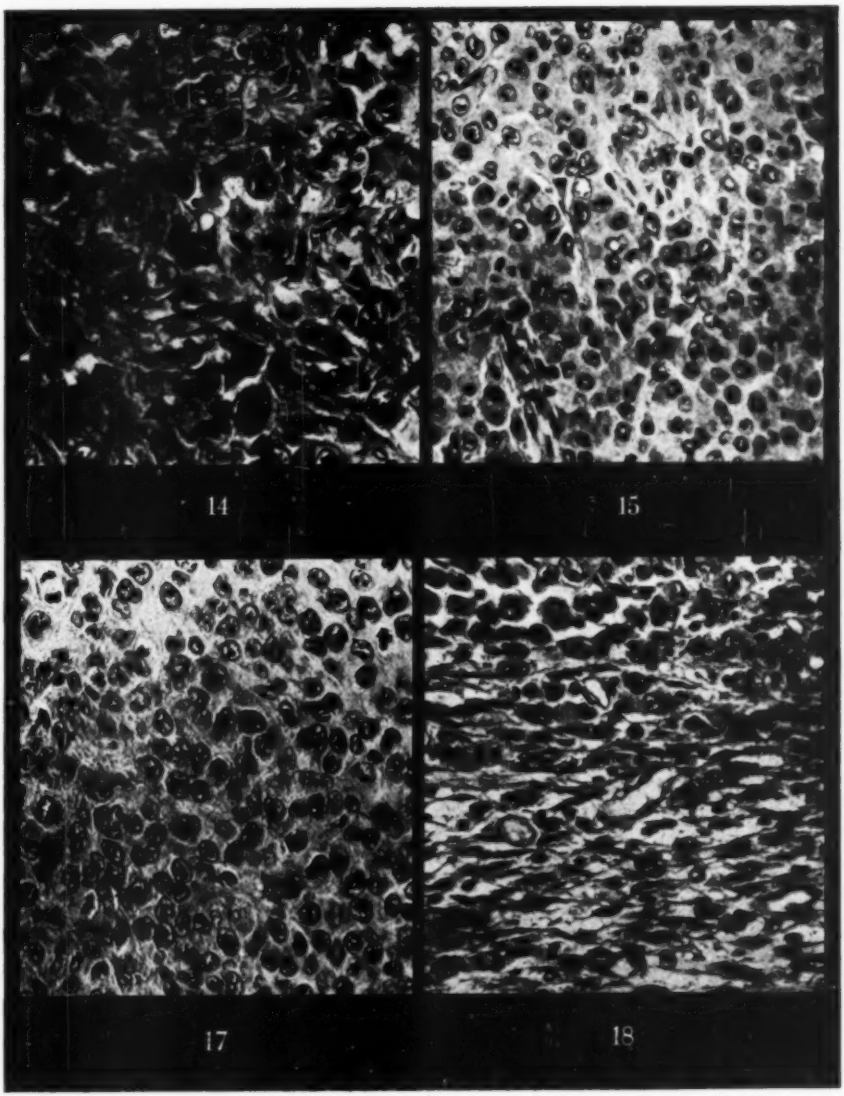

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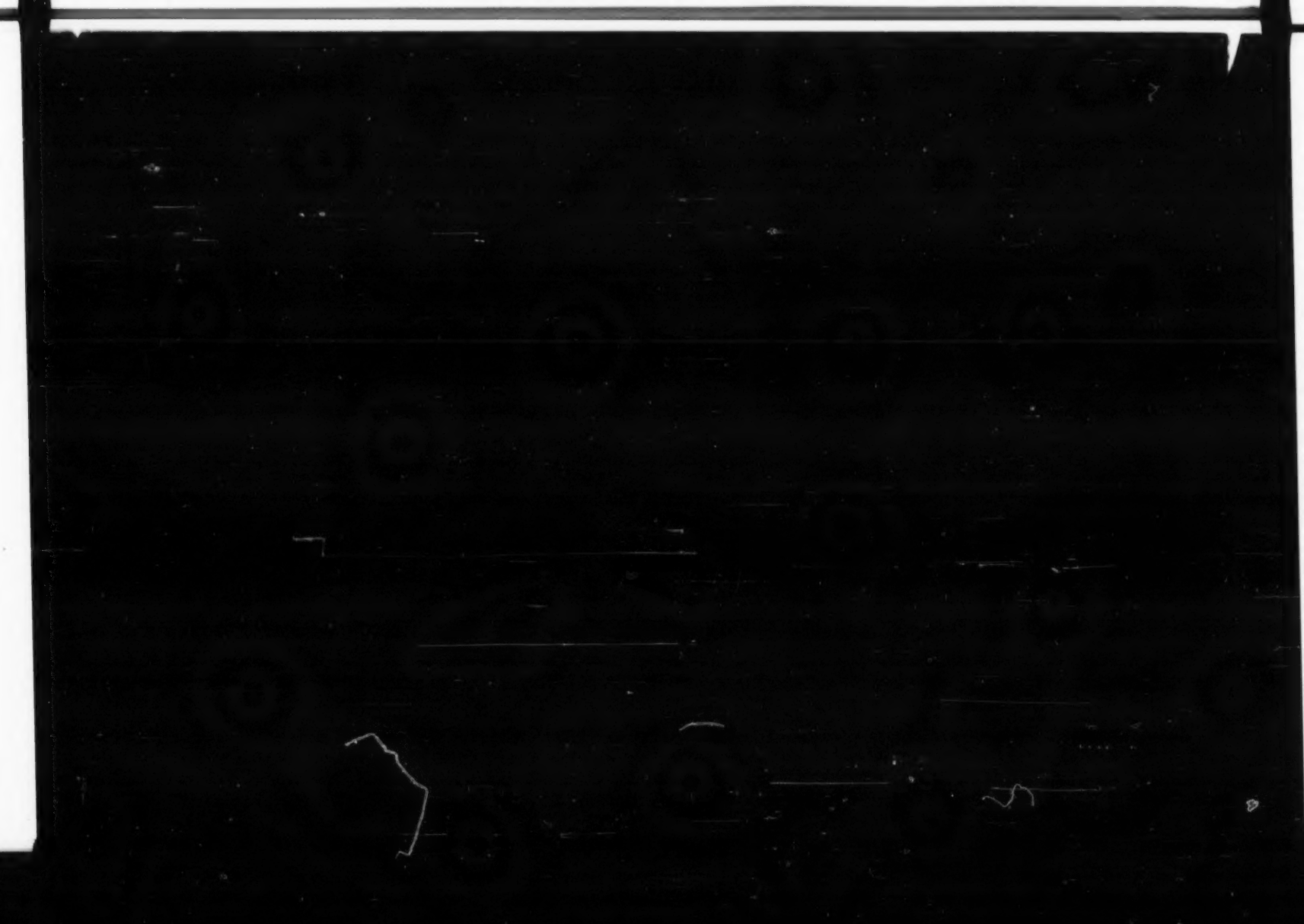

The cytological effects enumerated above result in a gradual transformation of the histological "organisation" of the Walker carcinoma (Fig. 10, 11, 12). The cellular structure gives place to a fibrous or "sarcomatous" tissue complex 4 or 5 days after treatment (Fig. 13). This consists of an extensive intercellular matrix of collagen in which dividing histiocytes and differentiating fibroblasts are in evidence. The number of tumour cells has greatly diminished, and in the place of the solid tumour parenchyma few large degenerating cells are left (Fig. 14). There is no measurable growth.

Our study shows that the primary cause of tumour inhibition is the destruction of a large proportion of active tumour cells. It has also been found that this process must be followed by gross histological changes if the aim is the permanent arrest of malignant growth. The histological transformation of the Walker carcinoma is very striking 4 days after treatment. The proliferation of those tumours cells which may recover from nuclear or cytoplasmic disturbances was found to be seriously hindered by the altered environmental conditions. Re-establishment of a solid tumour parenchyma does occur occasionally in the region of the connective-tissue capsule. The histological structure of this particular region is known to differ greatly from that of the tumour, and the different type of reaction to HN2 is presumably correlated with this.

In conclusion we wish to emphasize the important fact that 72 hours after HN2 administration a series of changes takes place which result in the complete transformation of the histological structure of the Walker carcinoma. No such effect was seen after treatment with 200 r X radiation.

Systemic effects and cytological injuries.

Experiments were next undertaken in which the whole body of the tumour-bearing animals was exposed to X rays to find whether systemic effects could influence the cytological response in the tumour itself. It has been shown that the cellular injuries brought about by X ray and HN2 differ greatly, but in view of the different techniques used, this observation does not necessarily indicate a corresponding difference in the efficiency of the two agents as tumour inhibitors. HN2 was administered by intraperitoneal injections, as a result of which not only the tumour but the whole organism came under the influence of the drug. The

TABLE III.—*The Effect of 200 r, 500 r Total Body Radiation and HN2 on the Body Weight of Five Rats 7 days after Treatment.*

	Control.		200 r total body.		500 r total body.		HN2.	
	Weight (in g.).	Weight gain in 7 days.	Weight before treatment.	Weight gain in 7 days after treatment.	Weight before treatment.	Weight gain in 7 days after treatment.	Weight before treatment.	Weight loss in 7 days after treatment.
	253	+17	235	+15	245	+12	225	−35
	272	+10	275	+10	270	+ 2	238	−31
	234	+12	235	+10	273	+ 8	210	−27
	210	+23	240	+20	250	+14	240	−30
	275	+15	242	+14	232	+ 8	235	−85*
Mean	249·8	+15·4	245·4	+13·8	254·0	+ 8·8	231·6	−37·6

* Animal suffers from diarrhoea.

X-ray dose on the other hand was delivered to the tumour only, the rest of the body being shielded from direct radiation.

In the experiments now to be described the whole body of rats was exposed to 200 r and 500 r, which are known to produce definite systemic effects of temporary duration. Both doses induce some systemic changes, e.g., in the cell components of the circulating blood.

Table III shows the effect of treatment on the body weight of the experimental animals. While there is no change when the rats are exposed to 200 r, the body weight was reduced when the dose was 500 r. This reduction begins 48 hours after irradiation and the amount lost may be considerable. The effect is, however, temporary ; the animals were already gaining weight again 7 days after treatment. In this respect the effect of HN2 is much more drastic than was observed after 500 r. The rats begin to lose weight 72 hours after HN2 administration, and some may lose one-third of the original weight. These particular animals usually suffer from severe diarrhoea and dehydration which may prove fatal. Our observation suggests that the toxic effects of nitrogen mustard affect the *whole* organism, and it is possible that they may also influence the cellular response in the tumour itself.

The cytological analysis in these experiments was restricted to animals whose whole body was exposed to 200 r from 72 to 168 hours previously. The data obtained are shown in Table IIB. It can be seen that the frequency of cells with mitotic injuries is the same as was found after localised X-ray treatment (Table I). No effect was observed on the histiocytes ; the tumour cell : histiocytes ratio was found to be similar to that of control tumours (Fig. 15). It was therefore concluded that the systemic effects of 200 r whole body radiation do not affect the cellular response of the tumour.

A similar experiment was carried out using 500 r whole body radiation. When the " LD_{50} " is used as a basis of comparison, this dose is estimated to correspond in efficiency to 1 mg. of HN2 per kg. body weight (intraperitoneal injection). While the cellular injury in the femoral bone marrow induced by 500 r was much more drastic than that produced by the HN2 " LD_{50}-equivalent " this X-ray dose was found to be still insufficient to bring about by way of the systemic effects the same amount of cellular and the same kind of histological changes which have been observed after HN2 administration. Though at present there is no way to determine the part which the general toxic effects of nitrogen mustard may play on the cytological and histological reaction pattern of the Walker carcinoma, such a possibility must clearly be considered.

Radiation reaction and histological changes.

Experiments were carried out with doses higher than 200 r applied direct to the tumour in order to find if irradiation can produce cytological and histological changes similar quantitatively and qualitatively to those observed after HN2 administration. Some information about the cytological effect of 300 r was already known from a previous investigation made by the senior author and his co-workers (Devik *et al.*, 1950). Those data have been extended in the present study and the results of analysis are compiled in Table IV. The data compare favourably with those obtained in experiments in which the tumour was exposed to 200 r (Table I). It was found that the cellular injuries decrease 12 hours after

irradiation and the effect of radiation is almost absent 144 hours after treatment. There was no change in the proportion of dividing histiocytes, and it appears that 300 r is not a sufficient radiation dose to induce histological alteration in the Walker carcinoma. The inhibition of tumour growth is of very short duration.

In another set of experiments the dose was increased to 1000 r (Table IV). During the analysis of the various tumour samples after exposure to 1000 r some interesting facts were brought to light. Dividing tumour cells are very rare 6 hours after treatment and the few cells found in mitosis show gross abnormalities. The chromosomes of these cells are " clumped " together or form many " sticky " bridges between the two telophase chromosome groups. Such abnormal " mitotic cells " are likely to be those cells which were in division at the time of irradiation. Owing to the difficulties associated with the interpretation of the mitotic disturbances no cytological analysis was carried out on the 6-hour tumour sample.

TABLE IV.—*The Frequencies of Injured Tumour cells from* 6 *to* 168 *hours after* 300 *r and* 1000 *r.*

300 roentgen (Devik *et al.* 1950 and additional data).						1000 roentgen.					
I.	II.	III. No.	III. %	IV.	V.	I.	II.	III. No.	III. %	IV.	V.
6	50	29	58·0	5·0	18.i.49	6	50	—	—	—	—
12	50	30	60·0	15·2	14.xii.48	12	50	21 (+6)*	42·0	12·0	20.i.52
24	50	22	44·0	19·6	15.xii.48	24	50	32	64·0	21·0	21.i.52
48	50	13	26·0	14·2	21.i.49	48	50	40	80·0	29·3	22.i.52
72	50	10	20·0	3·8	22.i.49	72	50	31	62·0	26·7	23.i.52
96	50	9	18·0	2·4	6.ii.49	96	50	12	24·0	18·4	24.i.52
120	50	5	10·0	6·5	20.x.51	120	50	7	14·0	20·6	25.i.52
144	50	3	6·0	3·6	21.x.51	144	50	9	18·0	6·0	26.i.52
168	50	3	6·0	3·8	22.x.51	168	50	3	6·0	7·5	27.i.52

* Cells showing clumped and sticky chromosomes.

Key to Table IV.

I. Time after treatment in hours.
II. Total number of cells in post-metaphase analysed.
III. Number and percentage of injured cells in post-metaphase.
IV. Percentage of cells in resting stage with micronuclei.
V. Date when animal was killed.

In the tumour 12 hours after treatment the rate of mitosis is still very low. The drastic reduction in the number of dividing cells is due to the high radiation dose which prevented cells at the end of the resting stage from entering mitosis. The delay in the developmental cycle may last 12 hours. This is indicated by the fact that in the 12-hour sample 6 cells with chromosome injuries were observed out of 27 in which the chromosome abnormality suggested that they were already in mitosis during irradiation. The frequency distribution of the injured cells observed in the various samples is a further indication that cell development has been retarded. While the highest proportion of injured cells is seen 12 hours after exposure to 300 r, the maximum damage was in the 48-hour tumour sample

after 1000 r (Fig. 16). It is also interesting to report that the rate of mitosis was found to be very low in all samples. This fact explains the reduced growth-rate of the Walker carcinoma which occurs after treatment with 1000 r.

The data obtained in these experiments were sufficient to show that the frequency of injured cells decreases with the increase of time between irradiation and fixation of the tumour sample for examination; thus 7 days after exposure to 1000 r only 6 per cent of the dividing cells were found to have chromosome injuries.

Special study has been made of the reaction of histiocytes in tumours irradiated with 1000 r. It was found that in all the samples under observation they make up only a relatively small fraction of the cell population which constitutes the tumour parenchyma. While there is, however, no histological alteration in the tumour proper (Fig. 17), a very definite change was noticed in the peripheral

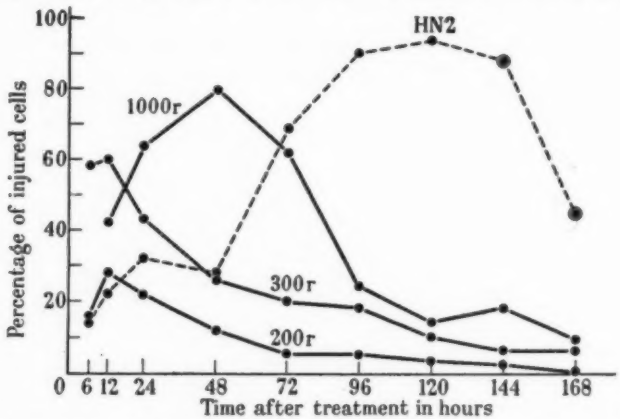

FIG. 16.—The frequency distribution of injured tumour cells after various doses of X-ray and HN2 treatment. The reduction in the number of injured cells between 144 and 168 hours after HN2 is exaggerated owing to the fact that some cells have undergone two mitoses since treatment.

zone (connective tissue-capsule). This particular region in the 96-hour sample becomes very rich in histiocytes; their increase is attributed to migration from the vascular system. In later samples the histological change (Fig. 18) resembles the initial stage of fibrous differentiation in the capsule of the Walker carcinoma described by Devik *et al.* (1950). The process, however, differs very much from the reaction which this carcinoma exhibits after HN2 administration. In the latter case histiocytes and fibroblasts increase throughout the whole tumour, which undergoes fibrosis. After a high dose of radiation "fibrosis" is restricted to the peripheral region, while the architecture of the tumour parenchyma remains unaltered. This explains why the resumption of tumour growth is more likely to occur after irradiation than after HN2 administration.

In view of these observations we may conclude therefore that not even the highest dose of X ray (1000 r) used in our experiments could bring about the same kind and same amount of histological changes as were seen after HN2 administration.

DISCUSSION.

The most important difference observed in our experiments between the cytological effects of X rays and HN2 is the fact that the maximum number of injured cells is found in the tumour samples examined early after irradiation and later after nitrogen mustard treatment (Fig. 16). The true significance of this observation lies in the relationship which it has to the primary reactions induced by the two agents.

The view is now widely accepted that during irradiation ionisation takes place in the cell, followed immediately by the formation of free radicals of the water molecules (Lea, 1946). This event represents the first step in the complex reaction-chain which in time leads to a definite biological phenomenon. Similarly there is no substantial delay between administration of HN2 and its primary reaction in the organism. The rate of hydrolysis of nitrogen mustard is extremely high in a biological system, and it has been shown recently by Batemann, Klopp and Cromer (1951) that it remains " active " only for a few minutes after intravenous injection. This is the main reason which allows us to compare the cellular effects of X ray and HN2 in tumour samples taken at the same time after treatment and to determine the relative sensitivity of the various stages of the mitotic cycle.

The measure of sensitivity of a cell is the frequency of chromosome injuries observed at a given time. The interval between treatment and fixing the cell for analysis indicates the cell stage during treatment. Using this criterion the radiation sensitivity of the mitotic cycle has been determined in pollen grains of *Tradescantia* (Koller, 1946). If our present data are interpreted on the same criterion, then we must draw the conclusion that tumour cells of the Walker carcinoma are most sensitive to X rays at the end of the resting stage or beginning of prophase, while they are least sensitive to HN2 at this stage. The difference in sensitivity is illustrated in Fig. 16.

The same phenomenon was first reported by Ford (1949), who compared the frequencies of chromosome breaks in *Vicia* after X rays and HN2. Revell (1952) employed a new and critical method in the study of chromosome effects of several substances (mustards, epoxides, etc.) and came to the same conclusion. The high sensitivity of tumour cells in the intermitotic stage to HN2 may be a general property of cells. The fact that X ray and HN2 initiate their effects at different stages strongly suggests that their mode of action is different. Thus, for instance, we may assume that X rays act directly on definite linkages of the already synthesized polypeptide structure of the prophase chromosome thread, while a chemical agent affects the resting stage chromosome-filament either before or during the process of synthesis. This aspect of the chemical effects is discussed in more detail by Revell (1952).

The higher sensitivity of the resting stage to the chemical agents has another implication. The non-dividing (intermitotic or " resting ") cells are believed to be functionally the most active cells, and often referred to as cells in the " metabolic phase " (Hughes, 1952). It is not unlikely that the degeneration of many non-dividing tumour cells which we observed after HN2 administration is due to a gross disturbance in this metabolic phase. If it is found that chemical agents act preferentially on cells in the resting stage, a new approach may possibly be opened to the chemotherapy of cancer.

Because the functional or morphological characteristics of cells are determined

in the resting stage it can be expected that cells of different types will show different degrees of response to HN2. Our experiment demonstrated such difference between tumour cells and histiocytes.

The study of the cytological behaviour of the Walker carcinoma under X ray and HN2 treatment has accordingly led to the conclusion that the mode of action of these agents differs, and there is other experimental evidence of different kinds to support this conclusion. By lowering the oxygen tension during irradiation, Baker and Sgourakis (1950) found a great reduction in the frequency of sex-linked lethals in *Drosophila*. No such reduction was observed when nitrogen mustard was used (Auerbach and Moser, 1951). In barley, mutations of particular genes were induced with a higher frequency by HN2 than was expected, indicating some specificity of action (Gustafsson and MacKey, 1948). No such phenomenon was demonstrated by ionizing radiation. The observation of Ford (1949) and Revell (1952), who found a higher breakage-frequency in particular chromosome and chromosome regions after HN2 administration, falls into the same category. Widner, Storer and Lushbaugh (1951) compared the effects of irradiation and HN2 on mitotic and intermitotic times in several normal and tumour tissues and found a significant difference which they attributed to the difference in the mode of action of these agents.

In view of the arguments presented above it would be a gross error to infer a similarity of the mode of action of HN2 and X rays, based on the similarity of some " end-products " (e.g., blistering of skin ; bleaching of pigmented hair, etc.). The latter are the result of a complex chain of reactions, which can be initiated by fundamentally different primary events. We believe this to be the case, and for that reason the objection is raised to labelling nitrogen mustard as a " radiomimetic " poison. Such an adjective, by implicitly emphasizing the resemblance of a series of biological phenomena which are " end-products," tends to obscure the important basic differences.

SUMMARY.

1. Cytological analysis of the femoral bone marrow and implanted Walker carcinoma of the rat has shown that up to 24 hours after treatment, 200 roentgens produce the same amount of chromosome injuries as 1 mg. of nitrogen mustard (HN2) per kg. body weight when it is given by intraperitoneal injection.

2. The number of tumour cells with chromosome injuries decreases 12 hours after irradiation (200 r), and damaged cells are almost absent in tumours 72 hours after treatment.

3. When HN2 is administered the proportion of injured cells increases with time, and 144 hours after injection about 90 per cent of the dividing cells exhibit chromosome injury.

4. There is a rapid increase in the number of dividing histiocytes and fibroblasts 72 hours after HN2 administration and many tumour cells in the resting stage undergo degenerative changes. The histological organisation of the Walker carcinoma is greatly altered 7 days after injection.

5. Body weight is greatly reduced after HN2 administration, which indicates a toxic effect on the whole organism, which plays an important role in the reaction of the tumour cells and tissue.

6. Such systemic effects as are induced by whole body irradiation with 200

and 500 r do not increase cellular injury and fail to induce any histological transformation of the Walker carcinoma.

7. A single dose of irradiation (1000 r) can induce histological alteration, but of a type very different from that produced by HN2. The latter involves the entire tumour parenchyma of the Walker carcinoma, while the former is restricted to the connective-tissue capsule around the tumour.

8. The number of tumour cells and histiocytes showing chromosome injury after HN2 administration differs in the same tumour, indicating a different sensitivity in the two cell types to nitrogen mustard.

9. The significant difference found between the frequency of distribution of injured cells suggests that cells at the end of the resting stage are the most sensitive to X rays and least sensitive to HN2.

10. The difference in behaviour shown by the Walker carcinoma after irradiation and after nitrogen mustard treatment indicates that the primary basic reactions initiated by these agents are fundamentally different.

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THE ANTIMITOTIC ACTION OF AN AROMATIC NITROGEN MUSTARD ON TISSUE CULTURES.

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THE nitrogen mustards have been shown to have a marked inhibitory action upon cell division, a property that warrants their inclusion in the miscellaneous group of the " mitotic poisons " (Loveless and Revel, 1949) and their use as chemotherapeutic agents in certain forms of malignant growth (Haddow, 1947). Some of the 2-chloroethylamines are mutagenic in Drosophila (Auerbach and Robson, 1947) and in Neurospora (Tatum, 1947), and more recently both the alkyl (Boyland and Horning, 1949) and the aryl (Haddow, Kon and Ross, 1948) derivatives have been shown to exhibit tumour-inducing properties.

The present work deals primarily with the effects of p-amino N-N-di-(2-chloroethyl) aniline hydrochloride on the number, duration and distribution of mitoses in tissue cultures during the first 1 to 3 days after exposure. An assay of this particular compound was considered desirable because of the eventual possibility of preparing coloured derivatives by diazotization of the unsubstituted amine.

MATERIAL AND METHODS.

The compound, also known as " R 128", has been synthesized by Everett and Ross (1949), who kindly supplied it for these experiments. For information on its properties and physical constants the reader may refer to their paper. These authors and Hanby, Bartley, Powell and Rydon (1947) deal with the behaviour of nitrogen mustards in solution.

In the course of this investigation it was observed that solutions of R 128 acquire a violet colour on standing for 4 to 6 hours. In order to determine whether this colour was due to oxidation, two aliquots of a freshly prepared solution were kept in Thunberg tubes, one of which was immediately evacuated and sealed and the other left open. The solution in contact with oxygen became coloured as usual, while that kept *in vacuo* remained colourless after 24 hours. This reaction possibly involves the biologically active groups since it coincides with inactivation (p. 189), and since colour-formation has also been observed in solutions of other nitrogen mustards of varied structure (Ross, 1949).

All the experiments were done on hanging-drop cultures of frontal bone osteoblasts of 12-days-old chick embryos. Though not very adequate for detailed cytological work because of the smallness and large number of the chick chromosomes, this material was found particularly suitable for quantitative treatment as regards variations in mitotic number and duration of the mitotic cycle. The culture medium consisted of 50 per cent chick plasma and 50 per cent chick embryo extract.

Three methods of examination were used :

I. *Mitotic counts in fixed and stained cultures.*

This method furnishes data on the total number of mitoses and on the number of cells undergoing each phase of division at a given moment, and therefore an indirect estimation of the delay or arrest at any stage of the cycle. The number of abnormalities can also be ascertained.

The cultures that showed the most abundant growth after two transplantations were selected, and the explants were cut in halves as closely similar in size as possible, under the dissecting microscope. Each pair was incubated for another 48 hours in normal medium to test the homogeneity of growth, and then the poison was added to one half and the other half was kept as a control in normal medium. Before addition of the drug the outgrowth was completely and carefully cut right down to the edge of the explant in both test and control cultures. This procedure ensures a thin outgrowth and gives a sharp transition between explant and growth zone, thus making it possible to count practically every mitosis.

To avoid loss of activity the R 128 solutions were always prepared immediately before use. It was found convenient to prepare comparatively concentrated mother solutions (1 mg. per c.c.) and then to make the dilutions to the required titre. These mother solutions were acid—as would be expected from the hydrolysis of a salt of a weak base and strong acid—and at this concentration the buffering action of Tyrode fluid was insufficient to raise the pH above 6, but on further dilution (of the order of 0·1 mg. per c.c.) it was found that solutions which were in the range of pH 6 in water exhibited a neutral pH in Tyrode. All the solutions used here were made up in Tyrode fluid.

The cultures were stained with Ehrlich's haematoxylin after hydrolysis in N hydrochloric acid for 8 to 10 minutes at 60° C. (Hughes and Fell, 1949).

Total mitotic counts were made in every culture. In order to facilitate counting the whole of the mitotic cycle was considered to be comprised within the period between the disappearance and the reappearance of the nucleoli. Exception was made, however, for those cells in which the nucleolus persisted in advanced prophase and, conversely, for telophases in which the nucleus was fully reconstructed before completion of cytokinesis. The line between pro- and metaphase was drawn just before the formation of the equatorial plate and that between ana- and telophase at the end of cleavage.

II. *Low power phase-contrast cinemicrography.*

This method permits a direct, though only approximate, measurement of the rate of division and duration of each phase in a group of cells.

For film recording the cultures were mounted in an air-tight chamber made with a metal frame between two coverslips and sealed with paraffin wax (Hughes and Swann, 1948). A few drops of R 128 solution were left in contact with the culture for 15 to 30 minutes at 37° and then withdrawn. Only young cultures—about 12 hours old—were used, since these had a thin outgrowth with practically no overlapping or cell migration deep into the plasma clot, thus ensuring a good phase-contrast and an immediate contact of the poison with the cells. After selecting a suitable zone of the outgrowth—not more than 200 cells, preferably all in one plane—filming was started immediately.

The apparatus, devised by Canti and lately improved by Hughes, consists of a phase-contrast microscope encased in a thermostatically controlled chamber and attached to a 35 mm. cine camera. The shutter and light source are synchronized by a clock movement and the time is printed on each frame. A slow-motion mechanism moves the microscope stage some 20μ per hour following approximately the rate of migration of the outgrowth. The records were made on 35 mm. film at a speed of 24 frames per hour.

III. *High power phase-contrast cinemicrography.*

Here a single cell was followed through mitosis on 16 mm. film and the films were analysed at a magnification of 2530 diameters. The chromosome movements can be seen in detail, and it is possible to measure accurately the duration of each mitotic phase.

The technical arrangements were similar to those for the low-power film recording, except that critical focusing and phase adjustment required constant observation, and the time interval between frames was much shorter. The actual taking of these films was done by Dr. A. F. W. Hughes with an apparatus of his own design. The cultures were kept in contact with the mustard solution for 15 to 30 minutes at 37° and examined from 10 to 120 minutes after withdrawal of the poison. The results obtained by this method should therefore be taken as representing only the immediate action of R 128; work had to be interrupted at this stage and a study of the effects after longer intervals could not be undertaken.

TABLE I.

Tests: "R128" solution incubated in—	Hours of incubation of the solution.	Number of cultures.	Growth after 24 hours.
Plasma	0	4	0
Serum	0	7	0
Plasma	1	4	0
Serum	1	5	0
Plasma	2	4	0
Serum	2	7	+
,,	3	5	+
,,	4	6	+ to ++
,,	6	6	+ to ++
,,	12	6	++
,,	24	6	++
Controls: Clot made up with embryo extract plus—			
Plasma	0	5	+++
Plasma + serum	0	10	++
,, ,,	12	6	++
,, ,,	24	6	++

No growth or very poor growth is indicated by 0, scanty growth by +, normal growth by ++ and exuberant growth by +++.

RESULTS.

Inactivation of R 128 in the culture medium.

In order to determine the rate of inactivation of the agent, a freshly made 10^{-4} g./ml. solution of R 128 in plasma was incubated at 37° for different periods of time and the culture coagulum was made up of equal parts of this solution and embryo extract, so that the final concentration of R 128 in the medium was 1:20,000 w./v. In a similar way a 2×10^{-4} g./ml. solution in serum was incubated and mixed with equal parts of plasma before planting to attain the same final concentration. Only serum solutions were used for incubation periods longer than 2 hours because of clotting of the plasma, the addition of heparin not being considered advisable.

The results are shown in Table I. Concentrations of the order of 10^{-4} are lethal or sublethal to the cultures, and therefore normal growth in the tests indicates inactivation of the poison. It may be noticed that inactivation begins between 4 and 6 hours, which coincides roughly with the appearance of colour in the serum solutions.

Mitotic counts in fixed and stained cultures.

Concentrated solutions of R 128 caused severe unspecific damage to all cells. Dilutions of 1 : 5000 to 1 : 20,000 wt./vol.—74×10^{-5} to 18×10^{-5} molar—stopped cell division almost completely ; growth was very scanty and only a few abnormal mitoses could be found. Resting cells showed nuclear pycnosis, vacuolation and widespread disintegration of both nucleus and cytoplasm. Most of the cultures exposed to these high concentrations died within the first day.

TABLE II.—*Effect of R 128 on the Number of Mitoses and Percentage Occurrence of each Phase.*

Expt. No.	Concentration in g./ml.	Hours of incubation.	Number of cultures.	Average number of mitoses per culture.	Prophase per cent.	Metaphase per cent.	Anaphase per cent.	Telophase per cent.	Abnormal mitoses per cent.
1	10^{-5} (A)	24	5	92·2	25·3	35·1	15·5	24·2	13·5
	10^{-5} (B)	24	5	118·0	22·5	36·8	19·4	21·4	14·6
	Controls	24	5	261·0	23·7	34·4	20·2	21·7	2·5
2	2×10^{-6} (A)	24	6	243·5	30·9	29·6	18·5	21·0	15·2
	Controls	24	6	522·3	26·8	28·7	19·7	24·8	2·3
3	10^{-6} (A)	24	6	253·6	33·2	32·5	16·4	17·9	17·3
	Controls	24	6	446·5	24·0	28·6	21·4	26·1	3·0
4	10^{-6} (B)	48	5	—	33·6	34·8	13·0	18·6	18·5
	Controls	48	5	—	27·2	27·6	18·8	26·4	5·0
5	10^{-6} (B)	48+24*	4	410	31·2	33·8	14·4	20·6	5·9
	Controls	48+24	4	409·5	28·6	29·0	14·6	27·8	2·4

(A) R 128 incorporated in the medium. (B) R 128 in Tyrode ; explants immersed for 30 minutes before culturing ; controls immersed in Tyrode alone for the same period.

* Outgrowth cut down completely after 48 hours and explants transferred to a mustard-free medium for another 24 hours.

Less concentrated solutions of the order of 1 : 50,000 to 1 : 100,000—74 $\times 10^{-6}$ and 37 $\times$ 10^{-6} molar—allowed indefinite survival and successful subcultivation, although the outgrowth was smaller than in normal cultures. The number of mitoses was reduced by more than 50 per cent and more than 10 per cent of them were abnormal (Table II). Most resting cells looked normal, but some showed signs of pycnosis, vacuolation and cytoplasmic disintegration.

Concentrations as low as 2 $\times$ 10^{-6} to 10^{-6} g./ml. (or 74 $\times$ 10^{-7} to 37 $\times$ 10^{-7} molar) still caused a marked reduction in the number of mitoses, ranging from 25 to 50 per cent in the first 24 hours (Table II). There was, however, almost no visible effect upon resting cells, and the area of the outgrowth was not significantly decreased by the treatment until some 48 hours after exposure (Fig. 21).

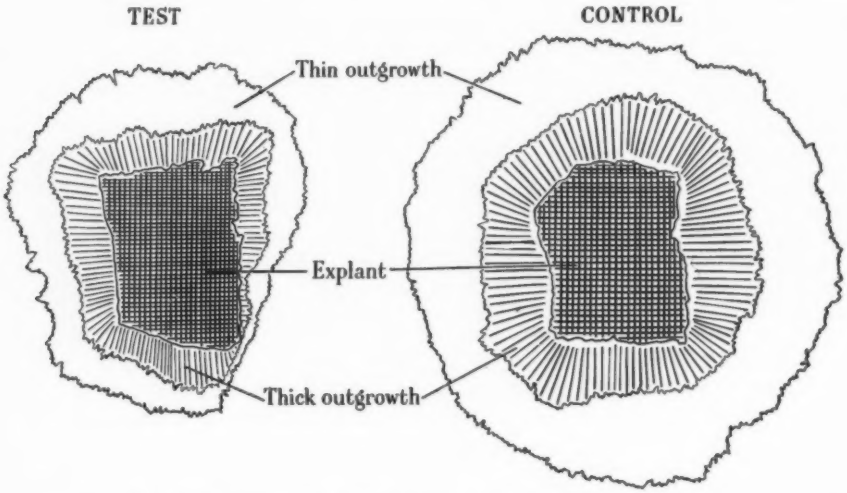


FIG. 21.—Difference in Area Between Control and Test Cultures.

48 hours after exposure to a 10^{-6} g./ml. solution of R 128 in Tyrode for 30 minutes. (Average of five pairs of cultures.)

	Explant.	Thick outgrowth.	Thin outgrowth.	Total outgrowth.
Total C.-T.=	—0·17 mm.²	6·18 mm.²	2·77 mm.²	8·95 mm.²
Mean C.-T.=	—0·03 „	1·24 „	0·55 „	1·79 „

After 48 hours' growth without subcultivation, total mitotic counts were found impracticable because of abundant cell overlapping, especially in the control cultures. At this stage the treated cultures showed no sign of recovery, but on the contrary there was a visible difference in the area of the outgrowth as compared with the controls. This was measured with the aid of a camera lucida as shown in Fig. 21.

The statistical analysis of the original figures was done in collaboration with Dr. D. V. Lindley using test "t" for P = 0·05, the χ^2 analysis of contingency tables and the analysis of the variance on the square root of the counts. The

effect of treatment on the total number of mitoses was significant in Experiments 1, 2 and 3 as well as the decrease in area in Experiment 4 but there was no quantitative residual effect after transferring the explants to a fresh medium in Experiment 5. The effect on the occurrence of each phase of mitosis was not significant except for a small increase in pro- and metaphase in Experiments 3 and 4.

The more frequent types of abnormalities caused by treatment with low concentrations of the mustard were clumping and scattering of chromatin in pro- and metaphase (Fig. 7, 8, 9), but ana- and telophase abnormalities were also found (Fig. 11 to 16), notably after 48 hours. The percentage of abnormal pro- and metaphases taken together was approximately 35 per cent after 24 hours and 20 per cent after 48 hours, while the number of abnormal ana- and telophases was only 4 per cent after 24 hours and reached 12 per cent after 48 hours.

In spite of the normal appearance of the resting cells, all the evidence obtained from the counts points to the fact that almost 100 per cent of the inhibitory action was exerted during interphase. The deviation in the proportions of pro- and metaphases found in some experiments accounts for less than 3 per cent and 1·5 per cent of all mitoses respectively. The phase distribution would still have remained unaltered, of course, if all stages had been retarded in exactly the same proportion, but the film analysis showed that this was not the case.

Low power phase-contrast cinemicrography.

The number of dividing cells per hour was counted throughout the duration of the experiment—12 to 24 hours. This was averaged and expressed as a percentage of the total number of cells present in the first frame (Table III).

TABLE III.

Concentration of R "128" to which the culture had been exposed.	Percentage of dividing cells per hour.
None (controls)	8·75
10^{-6} g./ml.	3·5
10^{-5} ,,	0·5
2×10^{-5} g./ml.	0·4
$0{\cdot}5 \times 10^{-4}$,,	0·05
2×10^{-4} ,,	0·00

Growth was partly inhibited by very low concentrations of nitrogen mustard (10^{-6} g./ml.), and was almost arrested with higher concentrations (2×10^{-4} and 5×10^{-5} g./ml.), when the cultures died within the first 12 hours. The toxic effect of such high doses is shown in Fig. 17*b*.

A good approximation of the duration of one individual mitosis can be obtained by the analysis of low power films. The chromosomes not being resolved, judgment of phase boundaries rests mainly on changes of shape and refringence (Fig. 18).

The average times for the stages of normal mitoses under these conditions were :

Pro + Metaphase, 20 minutes.
Anaphase, 5 minutes.
Telophase, 12 minutes.

No difference was found between the duration of 10 normal mitoses photographed during the first 5 hours and the last 5 hours of an experiment, nor between peripheral and central cells. Thus the possibility of delay in division being due to ageing or to cell crowding was excluded.

These phase-times were not significantly altered in the cultures treated with concentrations of 10^{-6} g./ml., although occasionally a cell might remain rounded up for more than double the normal time. With higher concentrations—10^{-5} and 2×10^{-5} g./ml.—the proportion of delayed divisions was increased, and in one film reached up to 20 per cent of the recorded instances. Delay was mainly confined to the " rounding-up " period, which was sometimes four times as long as the normal duration (80 instead of 20 minutes). Some cells, however, remained rounded up indefinitely, without showing any signs of anaphase movements. They probably represented the arrested metaphases observed in fixed and stained preparations and in high power phase-contrast films. In one culture treated with a 10^{-5} g./ml. solution a cell remained rounded up for 60 minutes, then elongated and continued thus for another 45 minutes, when it again rounded up. A similar case was recorded under high magnification (see below).

Six cells could be followed from one division to another in the control cultures. The intermitotic period varied from 5 to 20 hours, with an average of 10·6 hours. Cells in the middle had a longer resting stage than cells in the periphery of the outgrowth.

In the treated cultures no cell could be found to divide twice in one film (20 to 24 hours), but due to the scarcity of cell divisions it was not possible to decide whether the resting stage was prolonged or whether cells were prevented from dividing again.

High power phase-contrast cinemicrography.

The results have been reported elsewhere by Hughes (1950), and I shall only briefly summarize them here. As might have been expected from the observations reported in previous chapters, many of the individual cells examined behaved normally even in relatively high concentrations of the agent (Experiment 10, Table IV). Of 13 individual sequences recorded in cultures treated with concentrations ranging from 2×10^{-5} to 10^{-6} g./ml., 7 showed some signs of abnormality ; in 3 cleavage was imperfect, distorted or delayed, and in 4 metaphase was retarded or completely inhibited. One cell in which recording started when metaphase was well advanced (Experiment 11, Table IV) remained in metaphase with active bubbling for another 20 minutes and then the nucleus reconstructed without cell division (Fig. 19*a* and *b*). Another remained in metaphase throughout the duration of the film—1 hour 7 minutes 30 seconds—with random chromosome movements and very active bubbling, but no signs of spindle-formation. Other unrecorded instances of arrested metaphases were observed in cultures treated with concentrations of 10^{-5} g./ml. or higher. In every experiment the subsequent survival of the cultures was controlled by keeping other cultures identically treated in the incubator for 24 to 48 hours. Concentrations which killed the cultures were not used in these experiments.

It may be concluded, therefore, that under the conditions of the experiment many dividing cells escaped the immediate effect of the poison, and that when an effect was produced it usually appeared in metaphase and occasionally during cleavage.

TABLE IV.—*Some Sequences obtained with High Power Phase-contrast Cinemicrography.*

Expt. No. 10: Prophase to normal cleavage. Dilution: 2×10^{-5} g./ml.

Stage.	Frame No.	Time interval min.	Time interval sec.
Early prophase	0	16	24
Onset of metaphase	408	11	24
Onset of anaphase	692	5	12
Cleavage starts	722	2	18
End of cleavage	780		
Nuclei are reconstructed	1055	10	6

Expt. No. 13: Metaphase to normal cleavage. Dilution: 10^{-5}.

Stage.	Frame No.	Time interval min.	Time interval sec.
Onset of metaphase	0	9	18
Onset of anaphase	139	3	54
Cleavage starts	197		
End of cleavage	268	4	42
Nuclei are reconstructed	418	10	0

Expt. No. 5: Metaphase to delayed cleavage with reunion of daughter cells. Dilution: 2×10^{-6} g./ml.

Stage.	Frame No.	Time interval min.	Time interval sec.	Notes.
Late metaphase	0	2	48	—
Onset of anaphase	29	6	12	—
Cleavage starts	99	13	6	Incomplete cleavage; the daughter cells remain attached by a bridge of cytoplasm and then fuse again.
Nuclei are reconstructed	247	17	30	Mitochondria flowing from one pole to the other of the binucleate cell.
End of film	445	—	—	Cells still united. Two adult nuclei.

Expt. No. 11: Metaphase reconstruction (Fig. 19, *a* and *b*). Dilution: 2×10^{-5} g./ml.

Stage.	Frame No.	Time interval min.	Time interval sec.	Notes.
Late metaphase	0	3	36	Cell remarkably round.
Clumping	52	6	30	Chromosomes clump into small spherical mass.
Onset of anaphase ?	147	9	6	There is some attempt at anaphase but the chromosomes move centrally again.
Reconstruction starts	280	6	42	Chromosome contours are lost; the nucleus starts to reconstruct without cleavage.
Nucleoli appear	378	51	30	There is great increase in size of nucleus and cytoplasm, accompanied by intense bubbling. No cytokinesis ensues and the result is a single mononucleated giant cell, probably a tetraploid.
End of film	1129	—	—	

DISCUSSION.

The effect of R 128 in reducing the number of mitoses under the experimental conditions previously defined is very significant even with small concentrations. From the analysis of the effect of low concentrations it may be inferred that (*a*) this effect is exerted mainly during interphase; (*b*) the action upon dividing cells

is on the whole negligible ; when it exists, it appears chiefly in pro- and metaphase during the first 24 hours and begins to be significant in anaphase after this period ; (*c*) the interphase effect is not evidenced by morphological changes in the resting cells, while the mitotic effect is often, though not invariably, accompanied by cytological abnormalities ; and (*d*) the residual effect on growth is quantitatively not significant.

Lasnitzki (1940) found inhibition and retardation of prophase during the first 24 hours after exposure to small doses of X rays, in close similarity to what was found in some of our experiments. This immediate effect, as well as the metaphase abnormalities seen in fixed preparations and in the film recordings made shortly after the addition of the agent, are unspecific since they are also produced by mustard gas (Hughes and Fell, 1949) and by other "spindle inhibitors" (Hughes, 1950). Arrest of mitosis in interphase by nitrogen mustards has also been reported by Bodenstein (1947) in the embryonic ectoderm. The pycnosis and vacuolation of resting cells do not warrant discussion here since they were obtained with concentrations near or above the lethal. Similar toxic effects on chick fibroblasts were described with an aliphatic mustard by Fell and Allsopp (1949) in a wartime report.

The relative increase of anaphase abnormalities during the second 24 hours is of interest in connection with recent work by Loveless and Revell (1949), who found a significant increase in the rate of chromosome breaks and reunion in Vicia after a latent period following exposure to both aliphatic and aromatic nitrogen mustards, including R 128. Unfortunately the resolution obtainable with chick material is not sufficient to discriminate between chromosome fragments and small laggards (Fig. 14).

EXPLANATION OF PLATES.

FIGS. 1–16.—Material fixed with Maximow's fluid and stained in Ehrlich's haematoxiylin after hydrolysis in N HCl.

FIG. 17*a*–20*c*.—Phase contrast cine-camera photos of unstained living cells.

FIG. 1–6.—A normal mitotic sequence to show that cell division can proceed normally in the presence of low concentrations of R 128 (2×10^{-6} and 10^{-6} g./ml.). × 1300.

FIG. 7.—Shattering of chromosome material (10^{-5} g./ml.). × 1300.

FIG. 8.—Clumped chromosomes in late prophase or early metaphase (2×10^{-6} g./ml.). × 1300.

FIG. 9*a*.—Metaphase ; fusion of nuclear material (10^{-5} g./ml.). × 900.

FIG. 9*b*.—Metaphase ; clumping of chromosomes and spindle inhibition. Compare with phase-contrast picture of living cell (Fig. 19*a*) (10^{-6} g./ml.). × 1300.

FIG. 10.—Metaphase ; failure of congression on the plate (2×10^{-6} g./ml.). × 900.

FIG. 11, 12.—"Stickiness" in early and late anaphase (10^{-6} g./ml.). × 1300.

FIG. 13, 14.—Lagging chromosomes in early and late anaphase (10^{-6} g./ml.). × 900 and × 1300.

FIG. 15, 16.—Formation of a micronucleus in early and late telophase (10^{-6} g./ml.). × 900.

FIG. 17*a*.—Periphery of a living culture immediately after treatment with a concentrated solution (10^{-4} g./ml.) for 15 minutes. Cells are still unaffected. Approx. × 120.

FIG. 17*b*.—The same, 12 hours later. All cells are severely damaged. Approx. × 120.

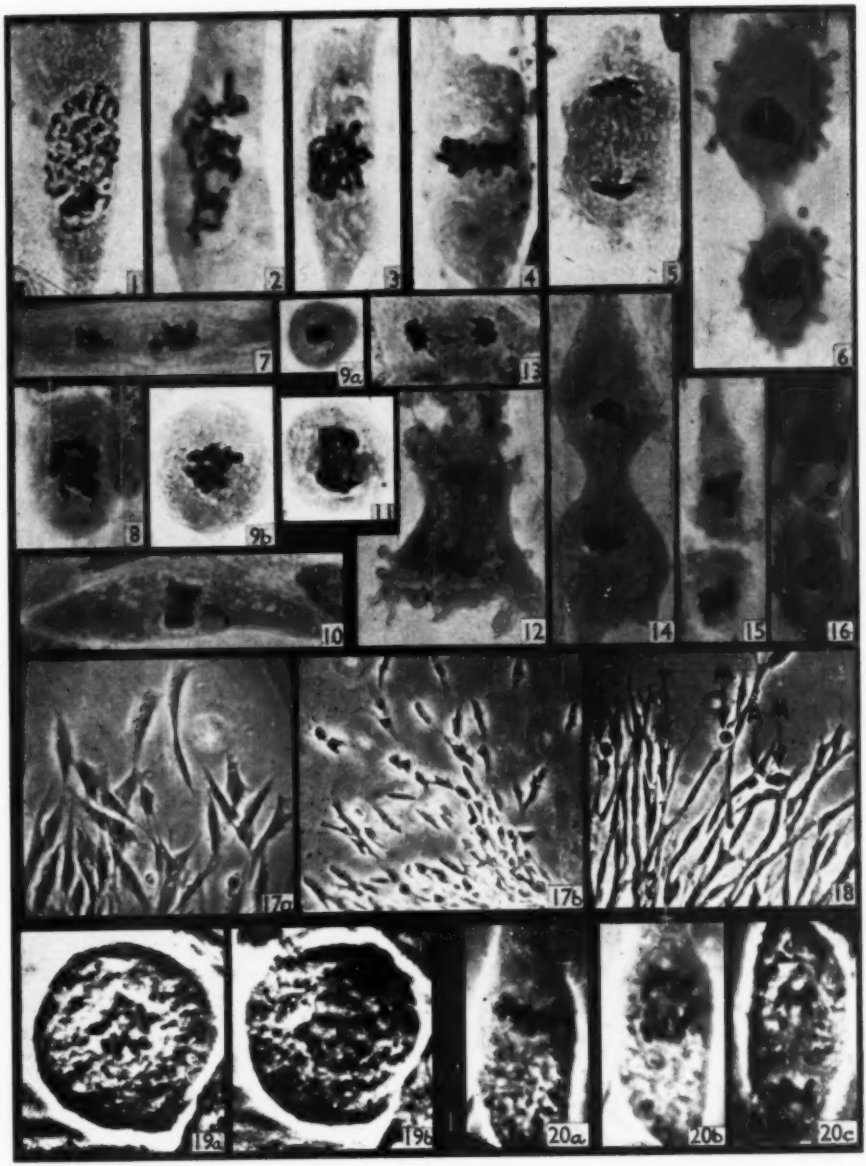
FIG. 18.—Edge of a control culture showing two metaphases (M), two anaphases (A) and a telophase (T). Approx. × 120.

FIG. 19*a*.—Spindle inhibition in a living cell 107 minutes after treatment (2×10^{-6} g./ml.). (For description see Table IV, Experiment 11.) Approx. × 1400.

FIG. 19*b*.—The same cell, 20 minutes later ; metaphase reconstruction without cleavage. Approx. × 1400.

FIG. 20*a*, *b*, *c*.—Normal anaphase movements in an untreated living cell for comparison. Approx. × 1400.

Figs. 19*a* and 19*b* are reproduced by kind permission of the *Quart. J. Microsc. Sci.* from the original pictures obtained by A. F. W. Hughes (1950).



Rinaldini.

Two types of effects have been observed in the present investigation, one functional, i.e., retardation of mitosis, and one structural, i.e., chromosome abnormalities, the former suggesting a disturbance of an enzymatic process (Boyland, 1950; Barron, Bartlett and Miller, 1948*a*; Barron *et al.*, 1948*b*), the latter being perhaps more readily explained by direct linkage of the mustard with a structural protein or nucleoprotein (Goldacre, Loveless and Ross, 1949; Loveless and Revell, 1949; Loveless, 1951; Butler, Gilbert and Smith, 1950; Gilbert, Overend and Webb, 1951). Although it is beyond the scope of this work to discuss the chemical aspects of the problem, it may be pointed out that these two current views on the mode of action of nitrogen mustards need not be mutually exclusive, since several enzymes have now been demonstrated in the nucleus and in the chromosomes and tryptophane-containing proteins have been isolated from chromosome material. One may venture to speculate that if a large part of the protein skeleton of the chromatid or the spindle fibre were contributed by the enzymes which control mitoses, by analogy with the role of adenosinetriphosphatase in the myofibril, then both the functional and the structural effects could respond to the same mechanism, or even, perhaps, be exerted on the same protein.

SUMMARY.

The action of p-amino-N-N-di (2-chloroethyl) aniline in concentrations of 2×10^{-4} to 10^{-6} g./ml. on the growth of tissue cultures was studied by mitotic counts and phase-contrast cinemicrography.

In every experiment a marked decrease of dividing cells was observed. High concentrations kill the cultures and produce gross cytological abnormalities in both dividing and resting cells. With dilute solutions a large proportion of cells are prevented from entering mitosis, but no pycnosis or vacuolation appears. This effect is exerted mainly during interphase. Pro- and metaphase abnormalities are conspicuous and ana- and telophase abnormalities rare during the first 24 hours, anaphase abnormalities increasing after this period. The duration of the first two phases is significantly prolonged in some cells. Cultures recover from these effects when transplanted into a fresh medium after 48 hours and hardly any residual effect can be detected. The possible implications of these findings are briefly discussed.

I wish to thank Professor J. F. Danielli for having introduced me to the subject, Dr. Honor B. Fell for her encouragement and valuable advice, Dr. A. F. W. Hughes for his help and guidance in the cinemicrography techniques, Dr. D. V. Lindley for his collaboration in the statistical analysis, and the technical staff of the Strangeways Laboratory, especially Mr. L. J. King, for their ready and efficient assistance.

I am indebted to the British Council for a grant that made this work possible.

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THE SUPRA-VITAL STAINING OF NORMAL AND MALIGNANT TISSUES WITH TETRAZOLIUM COMPOUNDS.

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THE staining of living tissues, either *in vitro* or in the form of freshly excised material, is a well recognised technique. Normally it is restricted to the identification of specific morphologic entities such as mitochondria. Attempts to determine intracellular biochemical features have been rather unsuccessful as far as living material is concerned, although successes have been achieved with fixed specimens. The availability of tetrazolium compounds now has rendered possible further attempts at the identification of intracellular sites of reduction.

Three compounds are generally available. In their normal state they form colourless solutions, but in the presence of suitable reducing groups they are converted to coloured formazans. The compounds concerned are :

2, 3, 5-triphenyl tetrazolium chloride [tetrazolium] giving a red colour on reduction.

2, 2′-(p-diphenylene)-bis-(3, 5-diphenyl tetrazolium chloride) [neotetrazolium] giving a red-purple colour on reduction.

2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride [iodotetrazolium] giving a purple-black colour on reduction.

Extensive use has been made of these substances for viability testing and for general staining purposes, but little has been reported on the specific details of the staining reactions achieved. It is the purpose of the present paper to present such details, together with the results of an investigation as to whether malignant tissues show any distinct differences to normal tissues in their reactions to these compounds.

MATERIALS AND METHODS.

The tetrazolium salts were used as solutions of concentrations varying between 0·1 and 0·5 per cent in normal saline. Tissues were taken from freshly killed animals or, in the case of human material, from freshly removed biopsy specimens. Small portions of tissue were teased out on a microscope slide in a drop of the staining solution and then squashed under a coverslip. This was ringed with wax to prevent drying during examination. After leaving for 5 to 10 minutes for the colour to develop the slides were examined with a standard microscope using direct illumination. To enhance contrast a light green filter was placed in front of the lamp.

RESULTS.

All three stains were taken up rapidly and evenly and colour formation was readily distinguished. The general features of the staining were the appearance of a pale diffuse coloration throughout the cell with a concentration of colour in the mitochondria, nuclear membrane, nucleoli and chromosomes of dividing cells. In the specialised case of striated muscle the colour was concentrated in the alternating bands, whilst structural details were picked out in paler shades. Irrespective of material or stain the general effect was as described above.

Specifically, no distinction in staining reaction could be determined between normal and malignant tissues. This is shown in the illustrations. Thus, normal human squamous epithelium (Fig. 1) is strictly comparable with cells from a human squamous epithelioma of the cervix (Fig. 2). Again, the staining of a " mast " cell (Fig. 3) and ciliated epithelial cells (Fig. 6) shows the same overall features as that of cells from transplanted mouse tumours (Fig. 4, 5, 7 and 8). No differences could be detected in the staining intensities of normal and malignant tissues, but within certain preparations of tumour cells it appeared that in the dividing nucleus the chromosomes of metaphase stained more deeply than those of prophase, anaphase or telophase. This finding is subject to verification as it is based on visual appearances only.

DISCUSSION.

Assuming satisfactory cellular penetration of the compounds used, the staining reactions achieved are in accord with biochemical data as to cell constituents. It is known that reducing groups occur throughout the cell and its formed structures, so staining should be expected to be general. The appearance of deeper staining in the mitochondria, nuclear membrane, nucleoli or chromosomes may not be entirely due to a greater local concentration of reducing groups, but could be partly the consequence of greater optical density giving an impression of deeper colour. In the case of muscle-fibres there does seem to be specific localization of reducing groups.

The actual reducing groups responsible for the colour formation are unknown, but there is some evidence that –SH groups are involved (Antopol, Glaubach and Goldman, 1948). If this is true then general staining is to be expected, since sulphydryl groups are essential constituents of many enzymes and proteins.

Considering that tumour cells differ but little from normal cells at a biochemical

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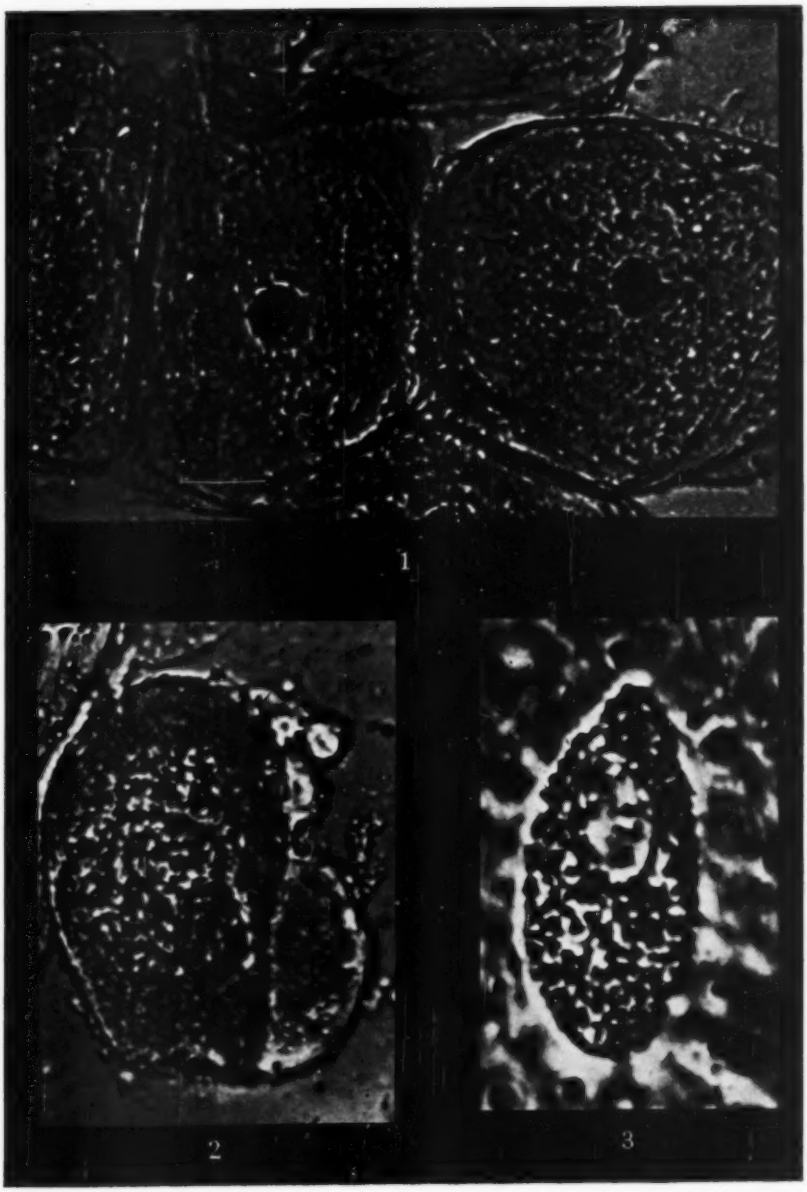
EXPLANATION OF PLATES.

FIG. 1.—Squamous epithelium from human cheek. Stained tetrazolium. × 960.
FIG. 2.—Cell from human squamous epithelioma of cervix. Stained iodotetrazolium. × 960.
FIG. 3.—" Mast " cell from mouse connective tissue. Note deeply stained granules. Stained neotetrazolium. × 2000.
FIG. 4.—Isolated resting cell from fast-growing transplantable sarcoma of RIII mice. Stained tetrazolium. × 2000.
FIG. 5.—Cell in metaphase from transplantable sarcoma of Strong A mice. Stained neotetrazolium. × 2000.
FIG. 6.—Ciliated epithelium from bronchus of rat. Stained iodotetrazolium. × 2000.
FIG. 7.—Resting cell from intraperitoneal transplant of sarcoma 378 in Strong A mice. Stained neotetrazolium. × 2000.
FIG. 8.—Resting cells and telophase from a transplantable mammary adenocarcinoma of Strong A mice. Stained iodotetrazolium. × 2000.
FIG. 9.—Striated muscle-fibres from mouse. Stained neotetrazolium. × 960.



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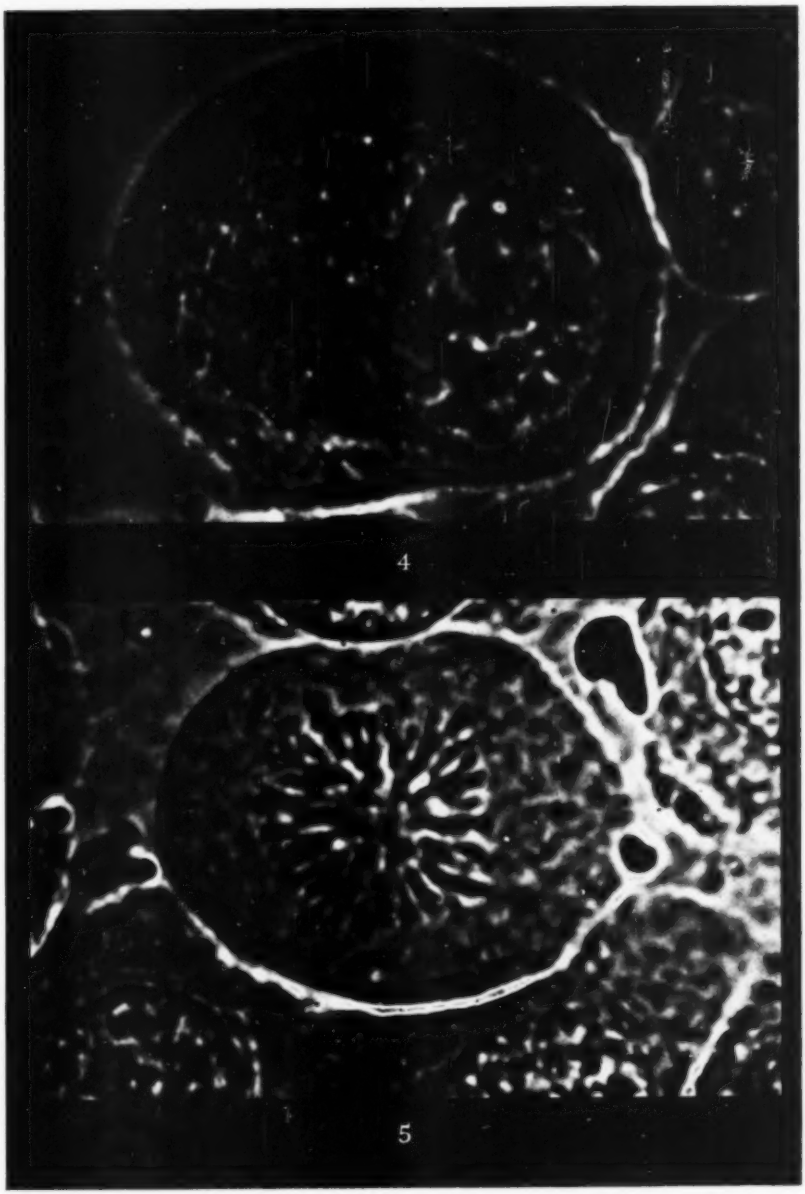

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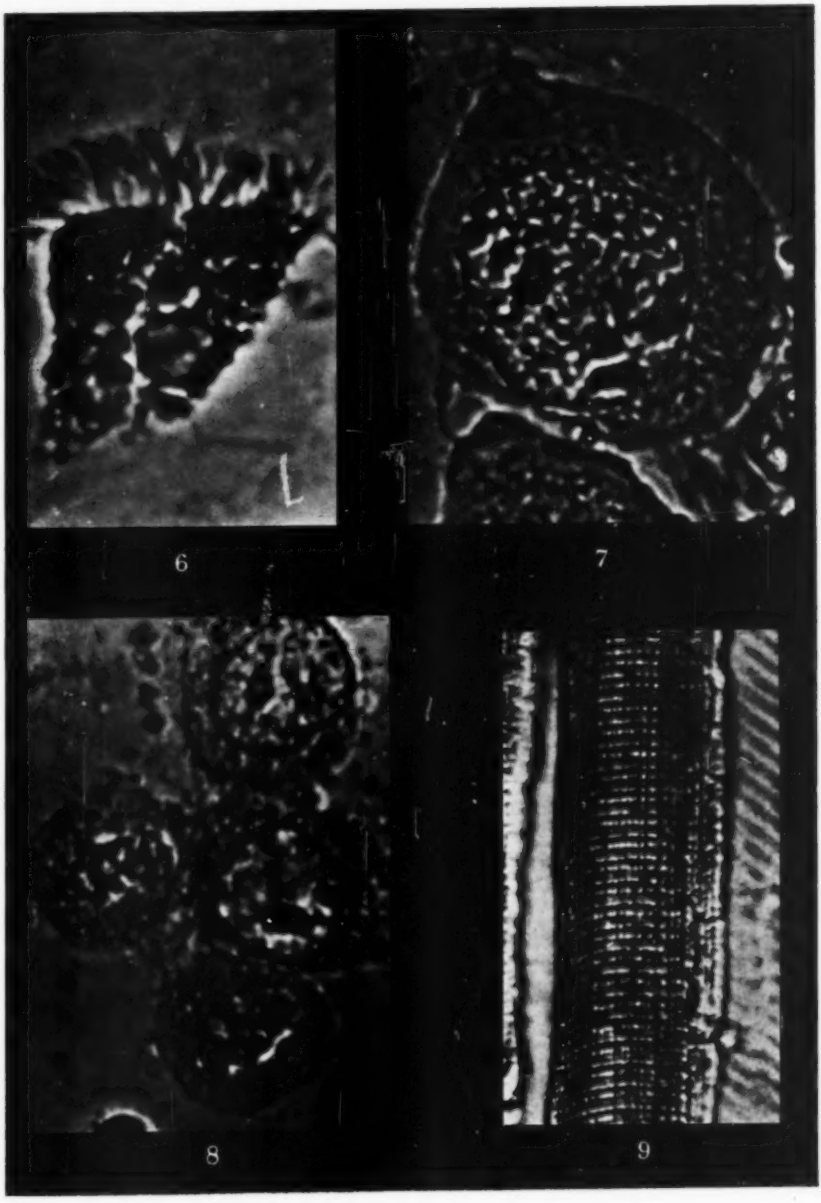

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level it is understandable that no basis for distinction on the grounds of staining reaction were found. This is, however, at variance with the experience of Straus, Cheronis and Straus (1948), who claimed that tetrazolium salts showed a selective affinity for certain types of malignant cells. This does not appear consistent with the chemistry of the processes involved.

In conclusion it may be said that as supra-vital stains the tetrazolium compounds are not particularly selective. On the other hand they easily give good general staining and a resultant picture which rather resembles that given by the phase contrast microscope. In this field they may have a use for teaching purposes or for rapid examination of small pieces of fresh tissue.

SUMMARY.

1. Teased out or smeared preparations of fresh tissues were mounted in saline solutions of tetrazolium salts.

2. Mitochondria, nuclear membranes, nucleoli and chromosomes showed deep staining against a diffusely stained background.

3. No evidence of any differences in staining reaction between normal and malignant tissues was found.

The author is indebted to Messrs. Pal Chemicals Ltd. (London) for the gift of the neotetrazolium and iodotetrazolium used in this work. Thanks are also due to Dr. P. Strickland of Mount Vernon Hospital for procuring the biopsy specimens, and to Miss J. M. Milton for technical assistance.

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THE PHOSPHORUS COMPOUNDS OF THE CELL NUCLEUS.

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THE three chief chemical constituents of the cell nucleus are deoxyribonucleic acid (DNA), a basic protein (histone or protamine), and a non-histone protein containing tryptophan (Stedman and Stedman, 1943, 1947, 1951; Mirsky and Pollister, 1946). It is now generally agreed that the cell nucleus also contains small amounts of ribonucleic acid (RNA) (Brachet, 1942; Davidson and Waymouth, 1944*a*; Euler and Hahn, 1946; Vendrely and Vendrely, 1948; Bergstrand, Eliasson, Hammarsten, Norberg, Reichard and Ubisch, 1948), which has been reported both in the nucleolus (Caspersson and Schultz, 1940; Brachet, 1942; Davidson and Waymouth, 1946; Pollister and Leuchtenberger, 1949*a*; Lagerstedt, 1948) and in the chromosomes (Mirsky and Ris, 1947).

In addition to DNA and RNA the nucleus also contains phospholipids and small amounts of other phosphorus compounds such as phosphoproteins (Davidson, Gardner, Hutchison, McIndoe, Raymond and Shaw, 1949; Davidson and McIndoe, 1949). In this paper such compounds are studied with the aid of radioactive phosphorus (^{32}P).

METHODS.

(a) *Isolation of nuclei.*

Calf thymus glands obtained from the slaughter-house as soon as possible after the death of the animal were homogenized in citric acid in a Waring blender or M.S.E. Nelco blender running at half speed, with mixing vessel surrounded by an ice jacket. The entire isolation procedure was carried out in the cold room or in a refrigerated centrifuge using the technique described by Mirsky and Pollister (1946), and was controlled in the later stages by frequent microscopic examinations.

The same procedure was used to obtain rabbit, rat and fowl liver nuclei (Fig. 1). The animals were killed by cervical dislocation or by exsanguination under ether anaesthesia and the livers immediately perfused with 0·9 per cent sodium chloride solution before being removed, chilled and homogenized. Nuclei were also prepared from the GRCH 15 tumour originally described by Peacock (1933).

For the preparation of fowl erythrocyte nuclei the method of Dounce and Lan (1943) was used.

In all cases the suspensions of clean nuclei were dried from the frozen state after small measured portions had been taken for dilution and counting in a haemocytometer. The dry weight of a given number of nuclei could thus be obtained. Photomicrographs of the isolated rat liver nuclei are shown in Fig. 1.

(b) *Radioactive phosphorus administration.*

^{32}P obtained from the Atomic Energy Research Establishment, Harwell, was administered as carrier-free inorganic phosphate by subcutaneous or intramuscular injection in doses of 100–500μc. per kg. body weight 2 or 4 hours before the animals were killed.

(c) *Analytical methods.*

(i) *Schmidt-Thannhauser* (1945) *technique* (*Fig.* 2).—Phosphorus compounds were separated by the method of Schmidt and Thannhauser (1945) as modified by Davidson, Frazer and Hutchison (1951). A weighed portion of dried nuclei

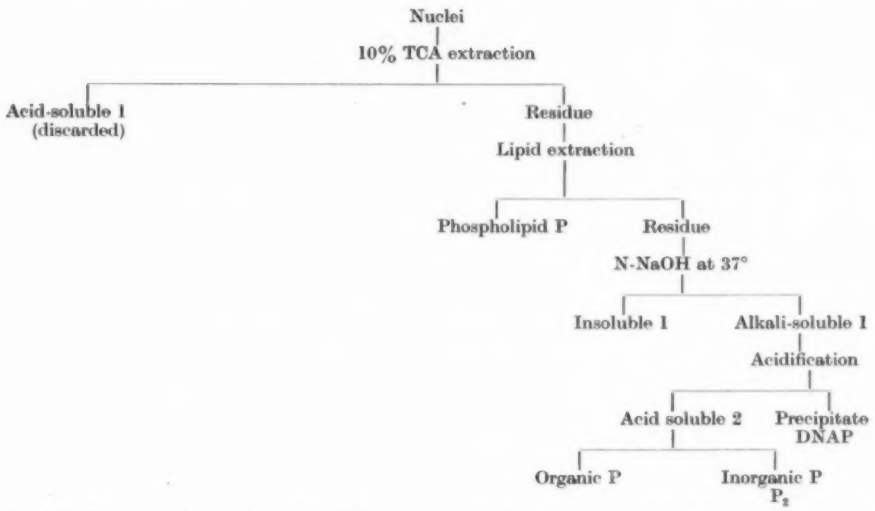

FIG. 2.—Scheme of separation of phosphorus compounds in nuclei (modified from Schmidt and Thannhauser, 1945).

was extracted three times with 10 per cent trichloracetic acid (TCA). The extracts were combined (acid soluble 1), but little attention was paid to this fraction since clearly much of the soluble P must already have been removed during the isolation of the nuclei. The extracted residue was treated with successive portions of acetone, ethanol, ethanol-chloroform (3:1), warm ethanol-ether (3:1) and ether. These extracts were taken to dryness and the residue taken up in chloroform to form the phospholipid fraction.

The dry nucleoprotein residue was incubated overnight at 37° with N NaOH to hydrolyse RNA to acid soluble nucleotides. A small amount of material failed to dissolve in warm alkali and was centrifuged down and washed with small amounts of N NaOH (Insoluble 1). The alkaline digest and washings were then neutralised with 5N HCl and treated with 0·5 vol. 30 per cent TCA. The precipitate of protein and DNA, after standing for 30 minutes in ice, was centrifuged down and washed twice with 10 per cent TCA. This precipitate (fraction

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" DNAP ") was dissolved in 0·1 N NaOH while the acid supernatant fluid and washings were combined as fraction acid soluble 2.

A portion of this fraction was then treated with Mathison's (1909) reagent and ammonium hydroxide in order to precipitate inorganic phosphate (fraction " P_2 " of Davidson, Frazer and Hutchison (1951), derived from " phosphoprotein "). The phosphorus which remained in solution was treated as organic phosphate (fraction " organic P ").

Fractions " acid soluble 2 " and " organic P " should contain phosphorus derived from RNA in the form of acid soluble nucleotides (RNAP). Pentose was therefore estimated in " acid soluble 2 " by the orcinol method of Mejbaum

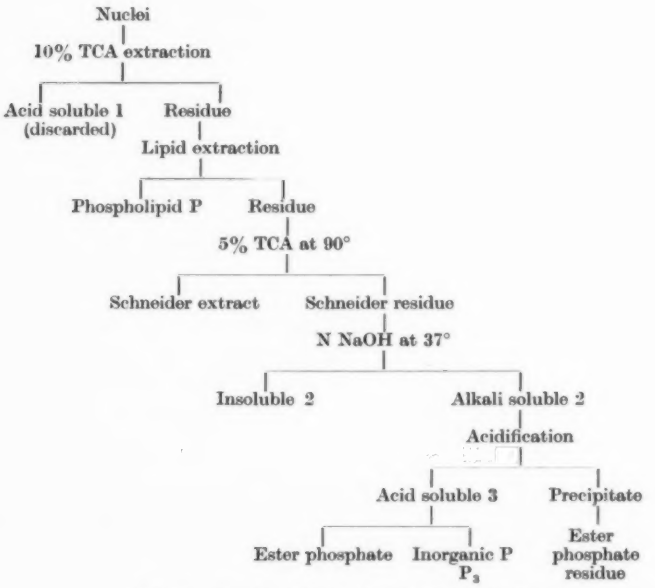


FIG. 3.—Combined Schneider and Schmidt-Thannhauser procedure.

(1939) and by the phloroglucinol method of Euler and Hahn (1946), using calibration curves made from a purified sample of yeast RNA, so that pentose could be read off directly in terms of ribonucleic acid phosphorus (RNAP).

Fraction " DNAP " should contain phosphorus derived from DNA only. Deoxypentose was therefore estimated by the diphenylamine method (Dische, 1930) as employed by Davidson and Waymouth (1944*b*), using a calibration curve prepared from a purified specimen of thymus DNA so that readings could be made directly in terms of deoxyribonucleic acid phosphorus (DNAP).

Phosphorus estimations were made on all samples by the method of Allen (1940).

The optical densities of fractions " acid soluble 2 " and " DNAP " were read in a Beckman Model DU spectrophotometer at 260 mμ. and 290 mμ. The differ-

ence in the readings was proportional to the amount of nucleic acid present as determined from standard solutions of RNA and DNA treated in the same way. The samples to be examined were diluted to contain less than 4 μg. nucleic acid P/ml. and were read against a reagent blank.

The radioactivity of each fraction was determined on a Type M6 liquid counter (20th Century Electronics) attached to a conventional scaling unit.

(ii) *The Schneider* (1945) *technique.*—A weighed portion of dried nuclei was extracted with TCA and lipid solvents, as described above. The extracted material was treated with 5 per cent TCA at 90° for 15 minutes to split off nucleic acids as acid soluble hydrolysis products. The residue was washed twice with 5 per cent TCA and was then dissolved in N NaOH to form the "Schneider residue". The combined TCA extract and washings formed the "Schneider extract". Phosphorus, pentose and deoxypentose were determined on both extract and residue, and ultraviolet absorption measurements made on the extract.

(iii) *Combined Schneider-Schmidt-Thannhauser procedure* (*Fig.* 3).—It was apparent at an early stage that although the "Schneider residue" might have been expected to contain only phosphoprotein P, its phosphorus content was in fact very much greater than that of fraction P_2. The "Schneider" residue clearly contained a mixture of phosphorus compounds, and it was therefore submitted to a Schmidt-Thannhauser separation by incubating in alkaline solution at 37° for 18 hours. An insoluble residue ("Insoluble 2") was centrifuged down and washed with dilute alkali. The alkaline digest with washings was then acidified with HCl and TCA as described above and separated into a precipitate ("ester phosphate residue") and an acid extract ("acid soluble 3").

Inorganic phosphate (P_3) was precipitated from the latter leaving an organic acid soluble phosphorus fraction ("ester phosphate"). Phosphorus and, where necessary, pentose and deoxypentose were determined in these fractions.

(iv) *Methods used in ionophoresis experiments.*—The separation of individual nucleotides from fraction "acid soluble 2" was carried out by the method of ionophoresis on paper described by Smellie and Davidson (1951) and Davidson and Smellie (1952). In such experiments the alkaline digest was prepared with 0·3 N KOH and was subsequently acidified with 60 per cent perchloric acid. The "acid soluble 2" fraction prepared in this way was brought to pH 3·5 by addition of alkali and dispensed on to a strip of Whatman 3 MM filter-paper (72 cm. × 7 cm.), which was moistened with 0·02 M citric acid-trisodium citrate buffer pH 3·5. To obtain adequate separation of the nucleotides the run was carried out at a potential gradient of about 10·7 volts/cm. for 18 hours. The ribonucleotides are preceded by components which run off the end of the paper under the conditions described above. To demonstrate them a separate run on a strip 57 cm. × 7 cm. was carried out at a potential gradient of 13·6 volts/cm. for 6 hours.

After a run the paper was dried and the bands located by the ultra-violet light method of Holiday and Johnson (1949) and marked lightly in pencil. Photographic records were made by the method of Markham and Smith (1949), and occasional autoradiographs were made by leaving the paper in contact with Kodak Industrex type D film for about 18 days.

The portions of the paper containing the bands were cut out and eluted according to Consden, Gordon and Martin (1947). The nature of each eluate

was determined by its position on the paper and its ultraviolet absorption spectrum. Total phosphorus and radioactivity were also determined.

RESULTS.

The results of a series of analyses are shown in Table I, in which the composition of bulk nuclei is given in terms of mg. P/100 g. dry weight. The phospholipid concentration is small, much below the value for whole tissue. The

TABLE I.—*Composition of Cell Nuclei from Various Sources and Specific Activities of Phosphorus in Liver Tissue 2 hours after administration of* ^{32}P.

P. Amount of phosphorus in mg./100 g. dry weight of nuclei.
C. Specific activity in counts/min./100 μg. P.

	Liver, rabbit.		Liver, rat.		Liver, rat (72 hr. fast).		Thymus, calf.	Erythrocytes, fowl bearing GRCH tumour.
	P	C	P	C	P	C	P	P
Phospholid	59	166	49	775	67	1640	30	101
Alkali soluble 1	1890	145	1770	319	1710	449	2810	2440
Acid soluble 2:								
Total P	310	846	327	1980	315	2260	195	203
RNAP by orcinol	224	—	263	—	266	—	131	88
by phloroglucinol	216	—	265	—	242	—	108	90
by UV absorption	—	—	276	—	274	—	177	—
Organic P	275	702	297	1560	265	2010	161	191
P_3	21	3380	14	3370	39	3430	23	5
DNAP by phosphorus	1610	16	1450	11	1410	15	2610	2240
by diphenylamine	1670	—	1320	—	1310	—	2480	2380
by UV absorption	—	—	1530	—	1380	—	2550	—
Schneider extract:								
Total P	1430	124	1540	297	1400	373	2320	2070
RNAP by orcinol	193	—	238	—	249	—	111	—
RNAP by phloroglucinol	202	—	234	—	228	—	101	100
DNAP by diphenylamine	1540	—	1440	—	1370	—	2460	2440
NAP by UV absorption	—	—	1440	—	1370	—	2010	—
Schneider residue:	405	303	288	563	306	697	447	283
Acid soluble 3	333	367	244	658	271	837	418	—
P_3	19	3850	30	2410	45	2830	37	—
Ester phosphate	289	94	215	216	221	208	366	—
Ester phosphate residue	66	83	44	157	51	201	48	—

presence of appreciable amounts of phosphorus in fraction "acid soluble 2" suggests that RNA is present in fairly large amounts in the cell nucleus, being more abundant in liver nuclei than in the nuclei of thymus or erythrocytes. Confirmation of the presence of RNA is given by the results of estimations of RNAP by the orcinol and phloroglucinol methods which show moderately good agreement, but figures obtained from measurements of ultraviolet absorption are rather more erratic. On the other hand the figure for "organic P" is frequently higher than that for RNAP. This is particularly evident in fowl erythrocyte nuclei, and may be due either to the presence of a variable quantity of an unknown phosphorus compound or to the leakage of a small amount of DNA into the "acid soluble 2" fraction. The latter explanation holds for calf thymus nuclei, in which ultraviolet absorption measurements give a figure considerably higher than that obtained by pentose estimation, but in all types of nuclei sub-

sequent experiments by ionophoresis on paper have conclusively proved the presence in the " acid soluble 2 " fraction of appreciable amounts of non-nucleotide phosphoric esters in addition to the expected nucleotides.

The difference between the figures for total P and " organic P " in the " acid soluble 2 " fraction is due to the presence of a small amount of inorganic phosphate (fraction P_2) which is derived from so-called " phosphoprotein." A similar fraction is present in whole tissue (Davidson, Frazer and Hutchison, 1951).

The amounts of DNAP estimated by the three different methods show moderately good agreement, although it is not unusual to find that, except in rabbit liver nuclei, the diphenylamine method tends to give a lower figure than do the other two methods.

Results obtained by the Schneider method are also shown in Table I. Although it might be expected that the Schneider extract would contain all the phosphorus derived from RNA and DNA, the figure obtained for this fraction is invariably

TABLE II.—*Mean Values for the Composition of the Single Cell Nucleus pg./nucleus.**

	Thymus, calf.	Liver, rabbit.	Liver, rat.	Liver, rat (72 hr. fast).	Liver, tumour-bearing fowl.	Erythrocyte, fowl.	Erythrocyte, tumour-bearing fowl.	GRCH 15 tumour, fowl.
		(Mean of 5.)	(Mean of 5.)	(Mean of 3.)		(Mean of 4.)	(Mean of 3.)	
Phospholipid	0·21	0·70	2·14	0·93	0·19	0·48	0·40	10·3
RNA by phosphorus estimation	0·45	1·21	2·88	2·13	0·55	0·25	0·14	6·38
by pentose estimation	0·33	1·04	2·56	1·84	0·48	0·20	0·14	7·50
by ultraviolet absorption	0·48	1·05†	2·68‡	1·89†	0·51	—	—	6·32
DNA by phosphorus estimation	7·30	6·57	9·65	9·85	2·91 (2·57†)	2·46	2·63	5·09 (4·90‡)
by diphenylamine reaction	6·90	6·79	8·70	9·27	2·64	2·22	2·51	4·30
by ultraviolet absorption	7·13	6·24†	9·69‡	9·76†	2·74	—	—	5·68
Nuclear mass	28·2	44·4	79·4	66·5	16·5	11·1	9·9	217

* pg. (picogram) = 10^{-12} g.
† Mean of 2.
‡ Mean of 4.

lower than the sum of " organic P " and " DNAP". At the same time the Schneider residue, which might be expected to contain only phosphoprotein P, invariably shows a much higher phosphorus content than could be accounted for by fraction P_2. This discrepancy was observed by Schneider (1945, 1946) for whole tissue and has been commented on by Davidson, Frazer and Hutchison (1951). That the Schneider residue contains several different types of phosphorus compound is made abundantly clear when it is submitted to a Schmidt-Thannhauser fractionation (Table I). The protein-bound inorganic fraction " P_3 " obtained in this way corresponds roughly in amount to fraction " P_2". It therefore represents the true " phosphoprotein " phosphorus, and the other phosphorus compounds in the Schneider residue are almost certainly derived from the non-nucleotide esters mentioned above, and from nucleic acid phosphorus which has failed to be removed in the Schneider extraction although the portions of the nucleic acid molecules containing the reactive sugars have been split off from the protein. This is clear from the fact that, in general, the figures for RNAP obtained by pentose estimation on the Schneider extract agree with those obtained

by pentose estimation on "acid soluble 2", while DNAP obtained by the diphenylamine method on the Schneider extract gives results which are comparable with those obtained from the Schmidt-Thannhauser DNAP fraction.

It is clear from Table I that dry preparations of calf thymus and fowl erythrocyte nuclei have a much higher DNA concentration and a lower RNA concentration than have liver nuclei, but a quite different light is shed on the results when they are expressed as amounts of each component per nucleus (Table II). The DNA content of the calf thymus nucleus (7·3 pg.) is of the same order as that found by Ris and Mirsky (1949), while the values for fowl nuclei (2·4 to 2·9 pg.) are very much lower and agree with the results of Davidson, Leslie, Smellie and Thomson (1950). The DNA content of nuclei from the GRCH 15 tumour of the fowl (5·1 pg.) is approximately double the value for normal fowl tissues. The figures for rat liver (9·65 pg.) are higher than those for other rat tissues, but are in agreement with the values found in a very extensive series of estimations at present being carried out in this department (Davidson, Heagy and Thomson, 1952, unpublished results).

TABLE III.—*Relative Composition of Nuclear Components Expressed in Moles P/10 Moles Adenylic P.*

	Thymus, calf.*	Liver, rabbit.	Liver, rat.†	Liver, rat.	Liver, normal. cock.	Liver, tumour-bearing cock.	GRCH 15 tumour, cock.
Acid soluble 2	170·4	66·3	64·9	67·4	83·1	80·0	80·7
Cytidylic acid	10·7	13·1	14·3	12·1	14·6	17·7	15·4
Adenylic acid	10·0	10·0	10·0	10·0	10·0	10·0	10·0
Component H	8·4	—	—	2·2	4·5	2·6	1·7
Guanylic acid	22·9	13·6	14·8	13·4	27·0	22·3	28·7
Uridylic acid	19·0	14·0	12·9	11·7	14·4	13·9	14·3
Component D	—	—	—	3·3	4·1	4·1	4·2
„ C	—	—	—		3·4	3·6	6·8
„ B	3·0	—	—	14·8	11·3	6·1	5·5
„ A	4·3	7·3	7·2				
Inorganic fraction	1·5	6·8	—	10·3	7·5	3·7	2·2
Organic fraction	2·8	0·5	—	4·5	3·8	2·4	3·3
Purine/Pyrimidine ratio	1·11	0·87	0·91	0·98	1·28	1·02	1·30

* The DNA derivative (see text) which lay between uridylic acid and component A and B accounted for 79 moles P in calf thymus nuclei.

† Mean of four preparations.

The RNA content of the individual nucleus is low in thymus and erythrocytes and is particularly high in the rat liver and in the GRCH 15 tumour. Nuclei from these last two sources have also a particularly high phospholipid content. The effect of a 72-hour fast is to reduce the amounts of phospholipid and RNA in rat liver nuclei and the nuclear mass, while the DNA content remains unchanged. The nuclear mass is particularly high in rat liver and in GRCH 15 tumour, but is low in the other fowl tissues.

It is clear from the results shown in Table I that fraction "acid soluble 2" contains phosphorus compounds other than simple ribonucleotides and inorganic phosphate. In an attempt to separate these compounds from the nucleotides, and the ribonucleotides from each other, the method of ionophoresis on paper was employed with the results shown in Table III.

In all the nuclei examined the four ribonucleotides cytidylic acid, adenylic

acid, guanylic acid, uridylic acid were identified by their absorption spectra. The order in which they occur on the paper is shown in Fig. 4, which is a photograph in ultraviolet light taken by the method of Markham and Smith (1949). In the material from one sample of rat liver nuclei each nucleotide was submitted to hydrolysis with perchloric acid (Marshak and Vogel, 1950), and the bases so liberated examined by paper chromatography by the method of Wyatt (1951). In each nucleotide only the expected base and no other was found. It is clear therefore that nuclear RNA is similar qualitatively to cytoplasmic RNA. We are indebted to Dr. G. Crosbie for the separation and estimation of the bases.

In different types of nuclei the proportions of the various nucleotides show only minor variations. Adenylic acid is least abundant and the purine to pyrimidine ratio is not from far unity. Using adenylic acid as a standard of reference (Table III) the most variable component is seen to be guanylic acid, which is particularly abundant in thymus nuclei, in the nuclei of the GRCH 15 tumour and in the liver nuclei of birds bearing this tumour. In most cases, particularly in thymus, a faint ultraviolet absorbing area was found on the paper between adenylic and guanylic acids (Fig. 4). The nature of this component, which we have termed H and which has properties very like those of adenylic acid itself, is as yet unknown, but it has been repeatedly detected in different kinds of nuclei.

In confirmation of the findings of Smellie and Davidson (1951) for whole liver tissue the nucleotides were found to be preceded by a fast-moving crescentic spot showing up faintly in an ultraviolet photograph (Fig. 5). This material, which we have designated component A, contains appreciable amounts of organic phosphate but inorganic phosphate is also present.

In early experiments with thymus nuclei and the nuclei of rabbit and rat liver component A alone was investigated, but subsequently, as the result of experiments with autoradiographs (see below), the whole area of the paper between component A and uridylic acid was examined by cutting it into sections corresponding to components B, C and D shown in Fig. 5. Component B lies immediately behind component A, from which it cannot readily be separated. It is probable that component B consists essentially of the inorganic moiety associated with component A. Component B should in fact correspond to fraction P_2 derived from phosphoprotein.

Component D lies immediately in front of uridylic acid and component C in the space between B and D; both C and D are organic in nature. It is clear therefore that fraction "acid soluble 2" contains in addition to the expected ribonucleotides at least four other components of which three are organic phosphates. These components are clearly visible in the autoradiographs shown in Fig. 4.

In material derived from calf thymus nuclei there was clear evidence that some breakdown of DNA had occurred with the liberation of fragments into the "acid soluble 2" fraction, for a spot of free guanine was found at the starting-point while the ribonucleotides were preceded by a massive ultraviolet absorbing spot containing a large amount of phosphorus (7·91 mole.P/mole. adenylic acid P, Table III). This spot contained adenine together with equimolecular amounts of reactive deoxypentose and phosphorus, and has been provisionally identified as an adenine-guanine dinucleotide from which guanine has been split off.

The extent of incorporation of ^{32}P into the various phosphorus fractions of

the cell nucleus during a 2-hour period is shown in Table I. The incorporation of ^{32}P into all fractions of fowl erythrocytes was so low as to make the results of little significance. The specific activity of the DNAP in rabbit and rat liver is very low indeed as compared with the cytoplasmic fractions, but by contrast the specific activity of the organic P fraction which contains ribonucleotides is very high indeed. In experiments in which we have compared nuclear RNAP and cytoplasmic RNAP the former has always shown a much higher rate of incorporation of ^{32}P than the latter (Davidson, McIndoe and Smellie, 1951). The specific activity of "organic P" is always less than that of fraction "acid soluble 2" from which it is derived. This is due to the presence in the latter of small amounts of "phosphoprotein" phosphorus (P_2), which has a very high activity indeed. As might be expected, the Schneider extract which contains breakdown products of RNA and DNA has a specific activity intermediate between that of "DNAP" and "organic P". The Schneider residue has an activity greater than that of the Schneider extract although much less than that of P_2, but when it is fractionated into its component parts, fraction P_3, which would appear to be true phosphoprotein phosphorus, corresponds both in amount and in activity to fraction P_2.

TABLE IV.—*Specific Activities (cts./min./100 μg. P) of Phosphorus in Nuclear Components. The nucleotides were obtained in a long run by ionophoresis on paper and the additional components in a short run.*

	Liver, rat.*	Liver, rat.	Liver, normal cock.	Liver, tumour-bearing cock.	GRCH 15 tumour, cock.
Dosage of 32 P (μg./kg. of body weight)	200	350	500	500	500
Acid soluble 2	1,030	5,560	11,600	4,180	2,960
Cytidylic acid	833	4,370	10,200	3,150	2,040
Adenylic acid	1,000	5,210	11,800	3,490	2,150
Component H	—	2,200	10,800	2,940	1,330
Guanylic acid	641	4,180	9,510	2,690	1,910
Uridylic acid	837	4,150	11,400	3,920	2,580
Component D	—	—	7,970	3,350	2,010
„ C	—	—	10,200	1,830	3,010
„ B	—	8,080	16,340	9,270	5,640
„ A	2,260				
Inorganic fraction of A + B	—	10,910	27,600	14,000	13,000
Organic fraction of A + B	—	1,320	1,470	1,850	563
DNAP	10	—	346	1,270	1,340

* Mean of 4 with adenylic acid taken as 1000.

A more detailed picture of the specific activities of the components of fraction "acid soluble 2" is given in Table IV, which shows the results of separation by ionophoresis on paper. The high specific activity of nuclear RNA is confirmed by isolation of the individual nucleotides. Although there is no great difference between the activity of the nucleotides, adenylic acid tends to show the highest activity. Components C and D have activities of the same order as those of the nucleotides, while the activities of components A and B together are considerably higher. When the combined components A and B are separated into organic and inorganic portions the inorganic fraction (which corresponds to P_2) is found to have a very high activity indeed, while the activity of the organic fraction is

lower than that of the nucleotides. The general pattern obtained from GRCH 15 tumour is similar to that for other tissues.

The presence of components A, B, C and D is shown clearly in the autoradiograph in Fig. 4. The nucleotides themselves together with component H, which is moderately active, show up in the autograph in Fig. 5, in which the areas of radioactivity are seen to correspond closely with the ultraviolet absorbing areas in the adjacent ultraviolet print.

Since the " DNAP " fraction has a low specific activity and is precipitated from a solution of much greater specific activity, it is possible that this fraction may be contaminated with small amounts of phosphorus of high activity although it is washed several times. It was therefore thought necessary to investigate the effect of dissolving the " DNAP " fraction in alkali and reprecipitating with acid. One such reprecipitation reduced the activity by about 40 per cent, but further reprecipitations proved to be unnecessary and indeed undesirable since they tended to promote degradation of the material. In all experiments in which ionophoresis was employed one reprecipitation of the DNAP fraction was used.

From Table I it is clear that the specific activity of the DNAP in rabbit and rat liver is very low indeed, and this is again evident in Table IV. The DNAP of the liver of the normal cock on the other hand has a much higher specific activity, while that of the DNAP in the GRCH 15 tumour, as might be expected in a rapidly growing tissue, is still higher and approaches the value for the ribonucleotides (Table IV). It is of interest that the specific activity of the DNAP in the liver of the tumour-bearing bird is higher than that in the normal bird. This is in agreement with the observation of Kelly and Jones (1951) that the presence of a tumour in any part of the body causes an increased incorporation of ^{32}P in the DNAP of liver and spleen.

DISCUSSION.

In examining results obtained from such a study as this consideration must be given to the degree of alteration which nuclei may undergo on isolation. Comparison of results obtained by chemical analysis and by the Feulgen staining technique applied to tissue sections suggests that no DNA is lost on isolation (Pollister and Leuchtenberger, 1949*b*; Ris and Mirsky, 1949), and confirmation of this view is to be found in the close agreement between the results obtained by different workers using different methods for the mean DNA content of the single nucleus. On the other hand fairly convincing evidence for the loss of protein from the nucleus during the process of isolation from aqueous media has been reported by Pollister and Leuchtenberger (1949*b*) and by Dounce, Tishkoff, Barnett and Frier (1950), although this is contested by Stedman and Stedman (1951). To what extent loss of RNA and other phosphorus-containing constituents may occur during isolation in citric acid is quite unknown.

It is clear from the results shown in Table I that, as might be expected, the bulk of the phosphorus in the nucleus is present as DNA, but the results of the Schmidt and Thannhauser procedure reveal considerable amounts of phosphorus in the " acid soluble 2 " fraction. That a large proportion of this fraction consists of the ribonucleotides produced by alkaline hydrolysis of RNA is evident from the results of pentose estimations by the orcinol and phloroglucinol methods, and the results of the ionophoresis experiments have conclusively established the presence in nuclear RNA of the four nucleotides which are found in RNA

from other sources. The presence of RNA in the nucleus has been suspected from histochemical tests with enzymes (Brachet, 1942; Davidson and Waymouth, 1946), from the results of ultraviolet absorption measurements (Caspersson and Schultz, 1940), and as the result of positive pentose tests on isolated nuclei (Euler and Hahn, 1946; Vendrely and Vendrely, 1948; Villela, 1949; Nash, 1949).

The present work not only demonstrates the presence of RNA in the nucleus but indicates its composition, which is seen from Table III to vary from one type of nucleus to another, the relative amounts of guanylic acid being particularly high in material from calf thymus and the tumours and livers of the tumour-bearing fowl. In a preliminary note Elson and Chargaff (1951) quote figures for the composition of rat liver nuclear RNA which are somewhat similar to ours, although their proportions of guanylic and uridylic acids are rather lower than those shown here. Marshak (1951) quotes figures for the molar composition of calf thymus nuclear RNA in which the proportions of cytosine and uracil are very low indeed. If his ribonucleotide fraction were contaminated with a DNA derivative such as we have found in our calf thymus preparations such low figures might be expected.

Comparison of the results of the molar composition of nuclear RNA with cytoplasmic RNA from the same type of cell reveals wide differences. Both in rabbit and rat liver and in calf thymus the cytoplasmic RNA is characterised by a higher guanine content than is found in the corresponding nuclear RNA (Chargaff, 1950; Marshak, 1951; Davidson and Smellie, 1952). Guanine is particularly abundant in the nuclear RNA of the GRCH 15 tumour and of the fowl liver, but it is also present in high relative proportions in the cytoplasmic RNA from the same material (Beale, Harris and Roe, 1950).

These differences in composition between nuclear and cytoplasmic RNA together with the much more rapid incorporation of ^{32}P by the former make it quite clear that the RNA of the nucleus is a separate entity, and is not merely derived from cytoplasmic fragments adhering to the isolated nuclei. The presence of component H, however, which would appear to be a nucleotide of ionophoretic mobility intermediate between that of adenylic and guanylic acids, has not so far been detected in any cytoplasmic RNA fraction examined in this laboratory. Components A, B, C and D on the other hand appear in the ribo-

EXPLANATION OF PLATES.

FIG. 1.—Isolated rat liver nuclei.

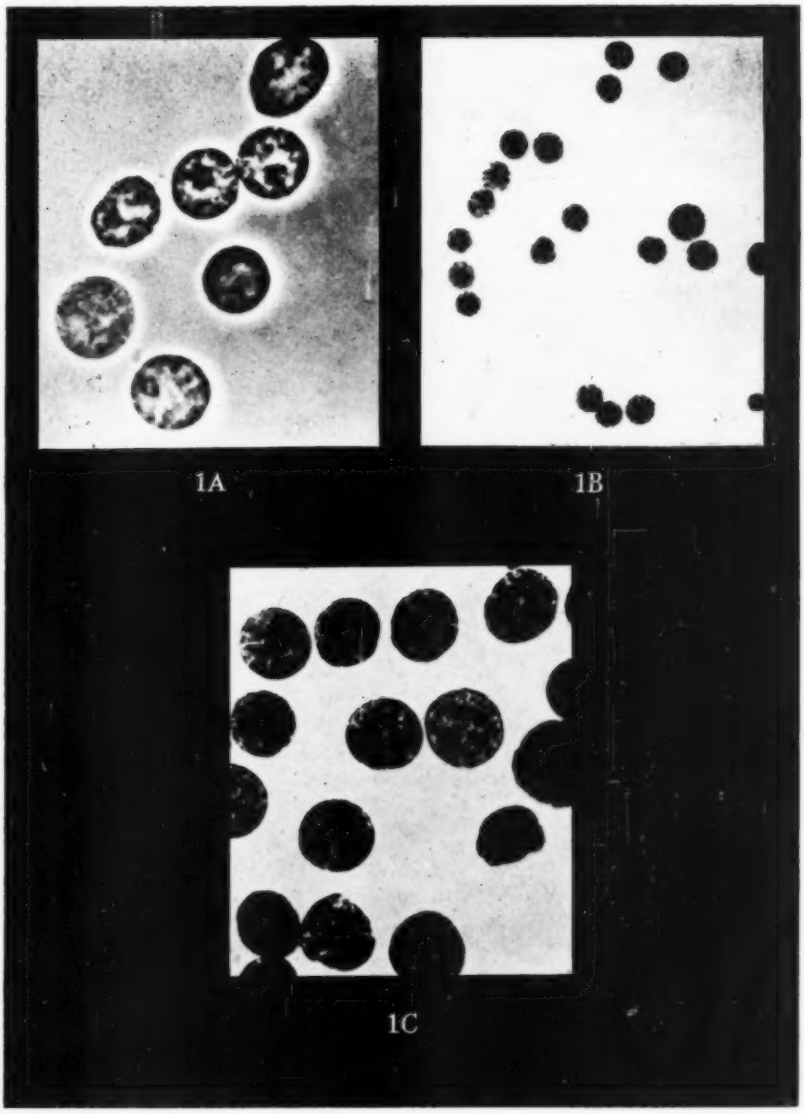
A. Photographed by phase contrast, magnification × 1600.
B and C. Photographed in ultraviolet light at 253·6 mμ.
B. Magnification × 500.
C. Magnification × 1600.

Ultraviolet prints (above) and autoradiographs (below) of the corresponding ionophoretic runs of the nucleotide fractions of rat liver nuclei.

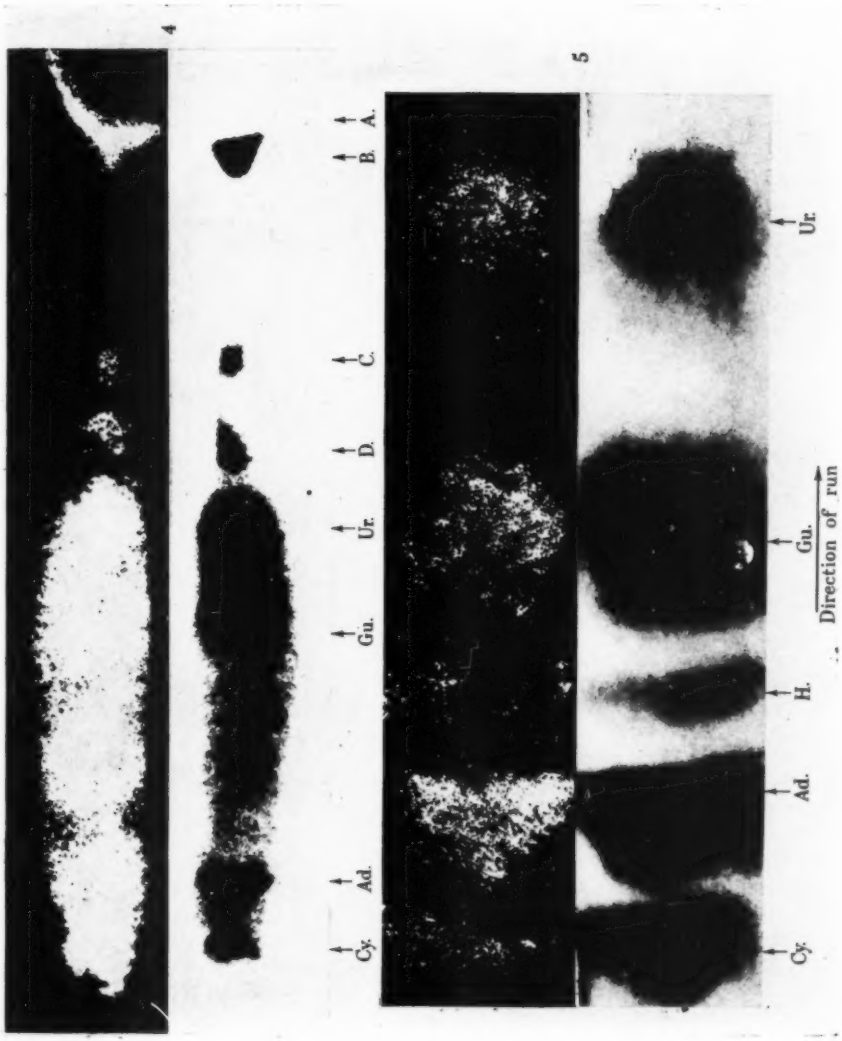
FIG. 4.—Short ionophoretic run. Component A shows up in the ultraviolet print in its characteristic crescentic shape. The nucleotides have not yet fully separated from each other.

FIG. 5.—Long ionophoretic run. The nucleotides are adequately separated and component H is visible between adenylic and guanylic acids.

Cy. = cytidylic acid. *Ad.* = adenylic acid.
Gu. = guanylic acid. *Ur.* = uridylic acid.



McIndoe and Davidson.



McIndoe and Davidson.

nucleotide fraction obtained from cytoplasmic materials by the Schmidt-Thannhauser (1945) separation procedure (Davidson and Smellie, 1952), although since the chemical nature of A, C and D is still unknown it is not possible to state whether the nuclear materials are identical with their cytoplasmic equivalents. Component B is essentially inorganic phosphate derived from " phosphoprotein " and corresponds to the fraction P_2 of Fig. 2.

Components A, C and D are organic phosphates, the presence of which is responsible, in part at least, for the difference between the " organic P " fraction in Table I and the RNAP by the orcinol reaction. The presence of such esters has already been suggested by Davidson and McIndoe (1949). It is clear that in nuclear tissue, as in whole tissue (Davidson, Frazer and Hutchison, 1951 ; Davidson and Smellie, 1952), the method of Schmidt and Thannhauser (1945) tends to give rather misleading results, since the RNA fraction contains, in addition to ribonucleotides, considerable amounts of non-ribonucleotide phosphate.

Incorporation studies with ^{32}P have revealed the rapid incorporation of the isotope into nuclear RNA and its slow incorporation into DNA in non-growing tissues.

Although nuclear RNA is much less abundant than cytoplasmic RNA it is more active, as has been shown by Marshak and Calvet (1949), Barnum and Huseby (1950), Jeener and Szafarz (1950) and Davidson, McIndoe and Smellie (1951) using ^{32}P and by Potter, Recknagel and Hurlbert (1951) using labelled orotic acid. The present studies show that this more rapid rate of incorporation of ^{32}P into nuclear RNA holds for all four nucleotides, which become labelled to practically the same extent.

The specific activities of components C and D are of the same order as those of the nucleotides 2 hours after administration of ^{32}P, but that of the inorganic phosphate fraction is much higher (Table IV). This component corresponds to fraction P_2 in Table I and is derived mainly from " phosphoprotein," although it may contain traces of inorganic phosphate originally present in the tissue. Its presence is responsible for the fact that the activity of fraction " acid soluble 2 " is higher than that of " organic P." The high activity of fraction P_2 in whole tissue has already been commented on by Davidson, Frazer and Hutchison (1951).

The results of the experiments on fowls confirm the observation of Kelly and Jones (1951) and Kelly, Payne, White and Jones (1951) in rats that the presence of a rapidly growing tumour in the animal body leads to an increased incorporation of ^{32}P into the liver DNAP. This effect is still unexplained and obviously requires much further examination, since it is associated in our experiments with the rather high specific activity of normal fowl liver DNA as compared with rat or rabbit liver DNA. The high activity for the DNA of the GRCH 15 tumour is in agreement with the results of other workers for rapidly growing tissues (Brues, Tracey and Cohn, 1944 ; Davidson and Raymond, 1948).

The results presented in Table II are of some interest in relation to the claims of Vendrely and Vendrely (1948, 1949) and of Mirsky and Ris (1949) that the DNA content of the cell nucleus is apparently constant for the cells of the different somatic tissues of any one species although there may be wide variations between one species and another. The implications of these observations have been discussed by Davidson and Leslie (1950*a*, *b*). The figures recorded in Table II

for the mean DNA content of the nucleus are in accord with those of other workers (for table see Davidson and Leslie, 1950*b*), and the results obtained by three different methods of estimation are of the same order, although the figures for the diphenylamine method are in general slightly lower than those obtained by phosphorus estimations or ultraviolet absorption measurements.

The mean DNA content of the rat liver nucleus (9·65 pg.), which is higher than for other rat tissues (about 6·5 pg. per nucleus) (Davidson, Heagy and Thomson, 1952, unpublished results), is apparently due to the occurrence of polyploidy (Swift, 1950). As might be expected, no loss of DNA from the rat liver nucleus occurs on fasting, although the RNA content falls. Too much stress however should not be laid on this latter observation, since the presence of minute amounts of cytoplasmic contamination could significantly alter the RNA content of the nucleus. It has already been shown (Davidson, 1947) that fasting causes a marked fall in the total RNA content of the rat liver, while the total DNA content is unchanged. This would of course be expected if the DNA content of the individual nuclei and the number of nuclei per liver were unaltered.

In the GRCH 15 fowl tumour the DNA content of the nucleus is approximately double that in the fowl erythrocyte or liver cell. The significance of this observation is as yet obscure, but a similar high value for tumour cells has been recorded by Klein (1951) for several mouse tumours. On the other hand normal values for the DNA content of tumour cell nuclei have been recorded for rat hepatoma by Mark and Ris (1949), Cunningham, Griffin and Luck (1950), Price, Miller, Miller and Weber (1950), and by Davidson, Heagy and Thomson (1952, unpublished results), for mouse lymphoma by Klein (1951), and for leukemic cells in man by Davidson, Leslie and White (1951).

SUMMARY.

1. The composition of cell nuclei isolated in bulk, from calf thymus, rabbit, rat and fowl liver, fowl erythrocytes and the GRCH 15 tumour is expressed in terms of dry weight and in terms of statistical amount per single nucleus.

2. The cell nucleus contains, in addition to DNA, appreciable amounts of RNA. By means of ionophoresis on paper it has been shown that nuclear RNA contains cytidylic acid, adenylic acid, guanylic acid and uridylic acid, but the ribonucleotide fraction prepared from nuclei by the Schmidt-Thannhauser technique contains in addition to these four nucleotides several other phosphate esters and a small amount of inorganic phosphate derived from "phosphoprotein."

3. The incorporation of ^{32}P into the nuclear components has been studied. The nuclear RNA incorporates the isotope much more readily than does cytoplasmic RNA. The rate of incorporation of ^{32}P into DNA is low in resting tissues.

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